# REGULATION OF THE ANTI-SENESCENCE FACTOR, TBX2, BY THE UV STRESS SIGNALLING PATHWAY AND THE MITOTIC CYCLIN DEPENDENT KINASES

by

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I, Amaal Abrahams, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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August, 2007

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# ABSTRACT

**Regulation of the anti-senescence factor, Tbx2, by the UV stress signalling pathway and the mitotic cyclin dependent kinases**

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The T-box gene family has achieved great prominence in the field of developmental biology because its members have been demonstrated to play important roles in embryonic development and mutations within several T-box genes are associated with a number of human congenital diseases. Several lines of evidence have also implicated members of the T-box gene family in cell cycle regulation and in cancer. Importantly, the highly related T-box factors, Tbx2 and Tbx3, can suppress senescence through repressing the cyclin dependent kinase inhibitors, $p21^{WAF1/CIP1/SDII}$ and $p19^{ARF}$. Both Tbx2 and Tbx3 have also been linked to several cancers primarily because their expression levels have been found to be deregulated in these cancers. However, despite the pivotal role that members of the T-box family play in a wide variety of biological processes, very little is known about the biochemical pathways that regulate their levels and transcriptional activity. In view of the detrimental consequences resulting from altered levels of T-box proteins, as seen both in developmental disorders and in certain cancers, the need to identify such pathways is important. The aim of this study was therefore to identify kinases that phosphorylate and regulate the levels and activity of Tbx2 with the view to understanding its role in cell cycle regulation and cancer. This study shows that the p38 stress mitogen-activated protein kinase, and the mitotic cyclin A/Cdk2 and cyclin B1/Cdk1, are direct regulators of Tbx2 both in vitro and in vivo.

It is possible that Tbx2 and Tbx3 may contribute towards the oncogenic process through their anti-senescence function, especially since a dominant negative form of Tbx2 induces senescence in melanoma cells overexpressing Tbx2. However, very little is known about whether Tbx2 is indeed regulated during replicative- or stress-induced senescence. In this study, using a breast cancer cell line known to overexpress Tbx2, the Tbx2 protein is shown to be specifically phosphorylated by the p38 kinase in response to stress induced by ultraviolet irradiation. Using site-directed mutagenesis and in vitro kinase assays, serine residues 336, 623 and 675 in the Tbx2 protein were identified as p38 target sites. These sites are also shown to be phosphorylated in vivo. Importantly, western blotting, immunofluorescence and reporter assays reveal that this phosphorylation leads to increased Tbx2 protein levels, predominant nuclear localisation of the protein, and an increase in the ability of Tbx2 to repress the $p21^{WAF1/CIP1/SDII}$ promoter. These results show, for the first time, that the ability of Tbx2 to repress the *p21* gene is enhanced in response to a stress-induced senescence pathway. This leads to a better understanding of the anti-senescence function of Tbx2.

The ability of Tbx2 to function as an anti-senescence factor, as well as its altered regulation being associated with certain cancers, suggests that its levels may need to be tightly regulated during the cell cycle. Indeed, the Tbx2 protein was previously shown to be regulated during the various phases of the cell cycle, peaking at G2. The changes in the Tbx2 protein levels did not match changes in *Tbx2* mRNA levels, suggesting that the protein may be regulated by post-translational modifications such as phosphorylation. This study shows that the phosphorylation status of Tbx2 is regulated during the cell cycle with levels of phosphorylation peaking in G2 and M, in mouse and human cells respectively. Phosphorylation was shown to be specifically mediated by the mitotic kinases as demonstrated in experiments when the mitotic kinase inhibitor, olomoucine, was included. This study provides data to suggest that Tbx2 may be regulated differently during the cell cycle in mouse and human cells. Using site-directed mutagenesis and in vitro kinase assays, Tbx2 was found to be specifically phosphorylated at serine residues 192 and 336 by cyclin A/Cdk2 and serine residues 336 and 342 by cyclin B1/Cdk1. These sites are also targets for phosphorylation in vivo since mutating them altered the phosphorylation status of Tbx2. Moreover, both cyclin A and B1 were shown to bind Tbx2 in vitro and in vivo and the minimal region required for binding was mapped to its DNA-binding domain. Importantly, immunofluorescence demonstrates that the Tbx2 protein localises specifically to the nucleus at G2; this translocation was shown to be blocked in the presence of olomoucine. Furthermore, western blot analyses and reporter assays showed that pseudo-phosphorylation by cyclin B1/Cdk1, but not cyclin A/Cdk2, leads to increased Tbx2 protein levels and an increase in the ability of Tbx2 to repress the $p21^{WAF1/CIP1/SDII}$ promoter. These results disclose, for the first time, that phosphorylation by cyclin A/Cdk2 and cyclin B1/Cdk1 of the Tbx2 protein regulates its activity. This data provide additional evidence to support a role for Tbx2 in the G2 and/or M phase of the cell cycle.

# CHAPTER 1: INTRODUCTION AND AIMS

## 1.1 Introduction

The T-box gene family encodes transcription factors that are defined by the conservation of the DNA-binding domain known as the T-box (Kispert and Herrmann, 1993; Bollag et al., 1994; Agulnik et al., 1995; reviewed in Papaioannou, 2001). This family plays an important role in embryonic development such as the specification of primary germ layers and cell fates during organogenesis (reviewed in Showell et al., 2004). Consistent with this role, mutations within several T-box genes are associated with a number of human congenital diseases. Recently, the T-box family members, Tbx2 and the very closely related factor, Tbx3 have also been implicated in cell cycle regulation and cancer. For example, their expression is upregulated in a number of cancers, including breast (Sinclair et al., 2002; Fan et al., 2004), pancreatic (Mahlamäki et al., 2002; Hansel et al., 2004), melanoma (Vance et al., 2005; Hoek et al., 2004), liver (Renard et al., 2007) and bladder (Ito et al., 2005). Both Tbx2 and Tbx3 function as transcriptional repressors (Carreira et al., 1998; Carlson et al., 2001; He et al., 1999; Sinha et al., 2000), and have been shown to prevent senescence through a mechanism involving their ability to repress the cyclin dependent kinase inhibitors $p19^{ARF}$ (Jacobs et al., 2000; Brummelkamp et al., 2002; Lingbeek et al., 2002) and $p21^{WAF1/CIP1/SDII}$ (referred to as p21) (Prince et al., 2004; Vance et al., 2005). Tbx3 can also cooperate with oncogenic Myc and Ras to induce cellular transformation and suppress apoptosis through repression of $p19^{ARF}$ expression and p53 protein levels (Carlson et al., 2002).

Even though members of the T-box family have been shown to play a pivotal role in a wide variety of developmental processes and to be implicated in tumourigenesis, very little is known about the biochemical pathways regulating the expression and activity of these transcription factors. Furthermore, while several studies have defined them as transcription activators and repressors there is a paucity of information regarding their target genes (reviewed in Naiche et al., 2005). The recent discovery of the involvement of T-box proteins in the cell cycle and cancer has intensified the need to elucidate their precise role in the regulation of these cellular processes.

This review will provide a general overview of the current literature on the regulation of Tbx2 during development, the cell cycle and cancer, and to introduce key areas of research pertaining to this thesis.

## 1.2 The T-box family

The T-box gene family is an ancient gene family as indicated by phylogenetic analysis. It is thought to have initally arisen in the common ancestor of metazoan organisms and that a genome wide duplication occurred over 600 million years ago in the early evolution of vertebrates (Agulnik et al., 1996). Following deviation from the common ancestor, further divergence by gene duplication occurred along individual evolutionary lineages: current phylogenetic analyses

have divided the family into five subfamilies, namely T, Tbx1, Tbx2, Tbx6 and Tbr1 (reviewed in Papaioannou and Silver, 1998). To date, 17 mammalian T-box genes have been identified (reviewed in Naiche et al., 2005). These genes are highly conserved in evolution with family members found in a wide range of organisms, including nematodes, frog, chick, mouse and human (reviewed in Papaioannou, 2001; Papaioannou and Silver, 1998 and Smith, 1999). The T-box comprises a region of approximately 200 amino acid residues, which can be located anywhere within the protein (reviewed in Smith, 1999) and specific residues within this region are highly conserved in all members of the family (reviewed in Wilson and Conlon, 2002). The T-box region does not possess sequence similarity to any known DNA-binding motif of other transcription factors (reviewed in Wilson and Conlon, 2002).

The prototype of the T-box family, Brachyury or T, is by far the most extensively studied and has been utilised as a model for elucidating the structure and function of other family members. The N-terminal half of the T protein contains the T-box responsible for binding to DNA, whereas the C-terminal region is required for transcriptional activation (Kispert and Herrmann, 1993; Kispert et al., 1995; Conlon et al., 1996). Brachyury binds to the 24 bp palindromic sequence AATTT(G/C)ACACCTAGGTGTGAAATT (Kispert and Herrmann, 1993; Müller and Herrmann, 1997) and in vitro studies have shown that it can bind as a monomer to a variety of differently spaced and orientated half sites, such as AGGTGTGAAATT, within this palindrome (Kispert et al., 1995; Conlon et al., 2001; reviewed in Papaioannou, 2001). In the past decade several family members have been identified in a variety of organisms and all members tested to date also recognise the above palindromic response element (reviewed in Naiche et al., 2005). The target specificity of T-box proteins is thought to depend on accessory proteins as well as the difference in spacing and orientation of half sites within the palindrome (Kispert et al., 1995; Conlon et al., 2001; Sinha et al., 2000; reviewed in Papaioannou, 2001).

T-box proteins can function as either transcriptional activators or repressors (Kispert et al., 1995; Conlon et al., 1996; Carreira et al., 1998; Stennard et al., 2003) with some T-box factors, such as Tbx20a (Tbx20 isoform) (Stennard et al., 2003) and Tbx2 (Paxton et al., 2002) possessing both activation and repression domains. Indeed, in vitro studies have revealed that Tbx20a and Tbx2 can act as either activator or repressor, depending on the cell context. Their role as transcriptional regulators is well documented but very little is known about the mechanism(s) by which T-box factors execute their function and how they themselves are regulated. It is however anticipated that the function of T-box proteins are regulated by protein-protein interactions with other transcription regulators and recent studies have revealed the importance of co-activators in the function of certain T-box proteins. Murakami et al. (2005) have identified the WW-domain-containing transcription regulator, TAZ (Kanai et al., 2000) as a potent co-activator of TBX5*. In this study the investigators show that TAZ physically associates with TBX5 and histone acetyltransferase (HAT) proteins to mediate TBX5-dependent gene activation.

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* The accepted convention will be used for human (TBX2) and when referring to both human and mouse, TBX2/Tbx2 will be used.

In another study, Tpit (Tbx19) was shown to drive expression of the pro-opiomelanocortin (POMC) gene in pituitary cells by recruiting the co-activators SRC/p160 and then co-operating with the homeoprotein, Pitx1 (Lamolet et al., 2001; Maira et al., 2003). While it is evident that the specificity of T-box factors for their target genes are due to variations between T-elements and differences in their regulatory domains, the answer as to how these transcription factors can possess such diverse roles in different cell/tissue types lies in the identification of accessory proteins constituting the transcriptional machinery needed for their activity.

Since T-box proteins are DNA-binding transcription factors, their localisation to the nucleus is essential in order to regulate their target genes. It is therefore anticipated that their subcellular distribution plays an important role in the regulation of their activity. Indeed, Tbx5 localises to both the nucleus and cytoplasm in tissue sections from developing chick hearts (Bimber et al., 2007), which suggests that its nuclear localisation is regulated. Importantly, in DiGeorge syndrome patients, the 1223delC mutation within Tbx1 not only prevents nuclear localisation but also abolishes the transcription activation property of the protein (Stoller and Epstein, 2005). In this study, when the 1223delC Tbx1 mutant was forced to localise to the nucleus by fusing the protein to the SV40 nuclear localisation signal (NLS), Tbx1 was still unable to activate transcription of a luciferase reporter vector, indicating the need of the mutated region for its transactivational activity. This study may provide an explanation for the haploinsufficiency of the TBX1 protein in patients with DiGeorge syndrome.

Nuclear localisation signals have been identified in several members of the T-box family including Tbx1 (Stoller and Epstein, 2005), Tbx3 (Carlson et al., 2001), TBX5 (Zaragoza et al., 2004), Tbx6 (Uchiyama et al., 2001) and Veg T (Zhang and King, 1996), and have been shown to play an important role in their nuclear localisation. For example, deletion of the TBX5 NLS, at amino acids 325-327 within its C-terminal region, is sufficient to prevent nuclear localisation of the protein (Zaragoza et al., 2004). The NLS in Tbx1 is also conserved in Brachyury and Tbx10 of mouse and human, and is sufficient to localise these transcription factors to the nucleus when expressed as GFP/β-GAL fusion proteins (Stoller and Epstein, 2005). It is important to note that NLS domains have not yet been identified for several T-box factors. This might suggest that these T-box proteins have novel NLS sequences or that they require binding to a NLS-containing protein(s) to chaperone them to the nucleus.

The T-box family has achieved great prominence in the field of developmental biology. Since the discovery of the first T-box factor, several studies have focused on the cloning of other family members and the determination of their spatial and temporal expression patterns during development. T-box factors are expressed throughout development where their function ranges from the specification of primary germ layers to the determination of cell fate during organogenesis (reviewed in Showell et al., 2004). Indeed, the importance of this gene family in development is underscored by the fact that mutations in several T-box genes are associated with a number of developmental defects (Table 1.1). In humans, a number of autosomal dominant and semi-dominant genetic syndromes have been linked to mutations in the T-box

**Table 1.1.** The phenotypic effects of known mutations in the, mouse and human T-box genes (Adapted from Naiche et al., 2005).

| Mouse gene; human gene | Human syndrome | Mouse heterozygote phenotype | Mouse homozygote phenotype |
|---|---|---|---|
| *T; T* | Not known | Short tail | Embryonic lethal, inhibits mesoderm induction |
| *Tbx19; TBX19 (TPIT)* | Recessive isolated ACTH deficiency | Normal | ACTH deficiency, pigment defects |
| *Tbx1; TBX1* | DiGeorge, craniofacial, glandular, vascular and heart abnormalities | Viable, thymus and vascular abnormalities | Neonatal lethal; craniofacial, glandular, vascular, and heart abnormalities |
| *Tbx10; TBX10* | Not known | Susceptibility to cleft lip and palate (Dancer: ectopic-gain-of-function) | Cleft lip and palate (Dancer: ectopic-gain-of-function) |
| *Tbx15; TBX15* | Not known | Normal | Craniofacial viable, malformations and pigment pattern alterations (droopy ear) |
| *Tbx18; TBX18* | Not known | Normal | Postnatal lethal, vertebral malformations |
| *Tbx20; TBX20* | Not known | Heart contractile function defects | Embryonic lethal, heart abnormalities |
| *Tbx22; TBX22* | X-linked cleft palate with ankyloglossia | Not known | Not known |
| *Tbx2; TBX2* | Not known | Normal | Embryonic lethal, heart and limb abnormalities |
| *Tbx3; TBX3* | Ulnar-mammary: hypoplastic mammary glands, abnormal external genitalia, limb abnormalities | Hypoplastic mammary glands, abnormal external genitalia | Embryonic lethal, yolk sac, limb and mammary gland defects |
| *Tbx4; TBX4* | Small patella | Reduced allantois growth rate | Embryonic lethal, allantois and hindlimb defects |
| *Tbx5; TBX5* | Holt-Oram, heart and hand abnormalities | Heart abnormalities, reduced viability | Embryonic lethal, severe heart malformations |
| *Tbx6; TBX6* | Not known | Normal | Embryonic lethal, somite abnormalities |
| *Tbr1; TBR1* | Not known | Normal | Olfactory bulb and cortical defects |
| *Eomes; EOMES* | Not known | Normal | Embryonic lethal, trophoblast and mesoderm failure |
| *Tbx21; TBX21 (TBET)* | Not known | Airway hyperresponsiveness, intermediate INF-$\gamma$ levels in Th1 cells | Airway hyperresponsiveness, no Th1 cells |

genes. For example, mutations in *TBX3* are linked to the autosomal dominant ulnar-mammary syndrome (Bamshad et al., 1997), while *TBX22* mutations are associated with the semi-dominant X-linked disorder, cleft palate with ankyloglossia (Braybrook et al., 2001). Dosage sensitivity or haploinsufficiency is postulated to be the mechanism by which mutations in T-box genes are responsible for these developmental defects.

Very little is known about the signalling pathways that regulate the expression of T-box factors during embryonic development and even less is known about the complex regulation of the expression profiles of T-box genes post-embryogenesis. Over the last few years there has therefore been a shift towards elucidating the regulation and function of T-box proteins at a molecular level. One approach has been to identify the signalling pathways that regulate T-box factors and another has been to determine their target genes. Members of the transforming growth factor-β (TGFβ), fibroblast growth factor (FGF) and Wnt signalling pathways are key players in the regulation of cellular proliferation, differentiation and patterning during early embryonic development and have been shown to both be regulators of, and to be regulated by, certain T-box genes (reviewed in Shi and Massagué, 2003; Thisse and Thisse, 2005; Logan and Nusse, 2004 and Showell et al., 2004). Due to the multiple roles of T-box factors during development, the following discussion will focus on Tbx2 and, where appropriate, the role of other family members.

## 1.3 Tbx2

Tbx2 belongs to the Tbx2 subfamily of T-box transcription factors which include Tbx3, Tbx4 and Tbx5 (Bollag et al., 1994; Agulnik et al., 1996). TBX2 is most closely related to TBX3 (12q24.1), whereas TBX4 and TBX5 are more closely related to one another. During development, Tbx2 has been implicated in several processes, such as coordinating the cell fate, patterning and morphogenesis of a wide range of tissues and organs including limbs, kidneys, lungs, mammary glands, heart and craniofacial structures. Furthermore, Tbx2 and the family member Tbx3 are also emerging as key regulators of the cell cycle and dosage sensitivity of these two T-box genes have been implicated in several cancers.

### 1.3.1 Tbx2 gene and protein

In mouse Tbx2 is located within chromosome 11 (Bollag et al., 1994) and in humans, TBX2 has been mapped to chromosome 17q23 (Campbell et al., 1995). The T-box region of the human TBX2 transcription factor shares 90% DNA sequence homology and 96% peptide sequence homology with its mouse counterpart (Law et al., 1995). Both human and mouse Tbx2, which span 3.378 kb and 2.281 kb respectively, possess 7 exons (Fig. 1.1). In mouse, *Tbx2* is expressed in adult heart, lung, kidney, ovary and in cells of the melanocyte lineage (Bollag et al., 1994; Carreira et al., 1998). *TBX2* is expressed in a wide variety of tissues including fetal kidney and lung as well as in a number of adult tissues such as kidney, lung, placenta, ovary, prostate, spleen, testis, breast, heart, thymus, intestine and polymorphonucleocytes (Law et al., 1995; Campbell et al., 1995). While loss or altered expression of *Tbx2* has not been associated with any developmental syndrome, mutational studies in mice have shown that although *Tbx2* heterozygotes exhibit a normal profile, homozygote mutants die *in utero*

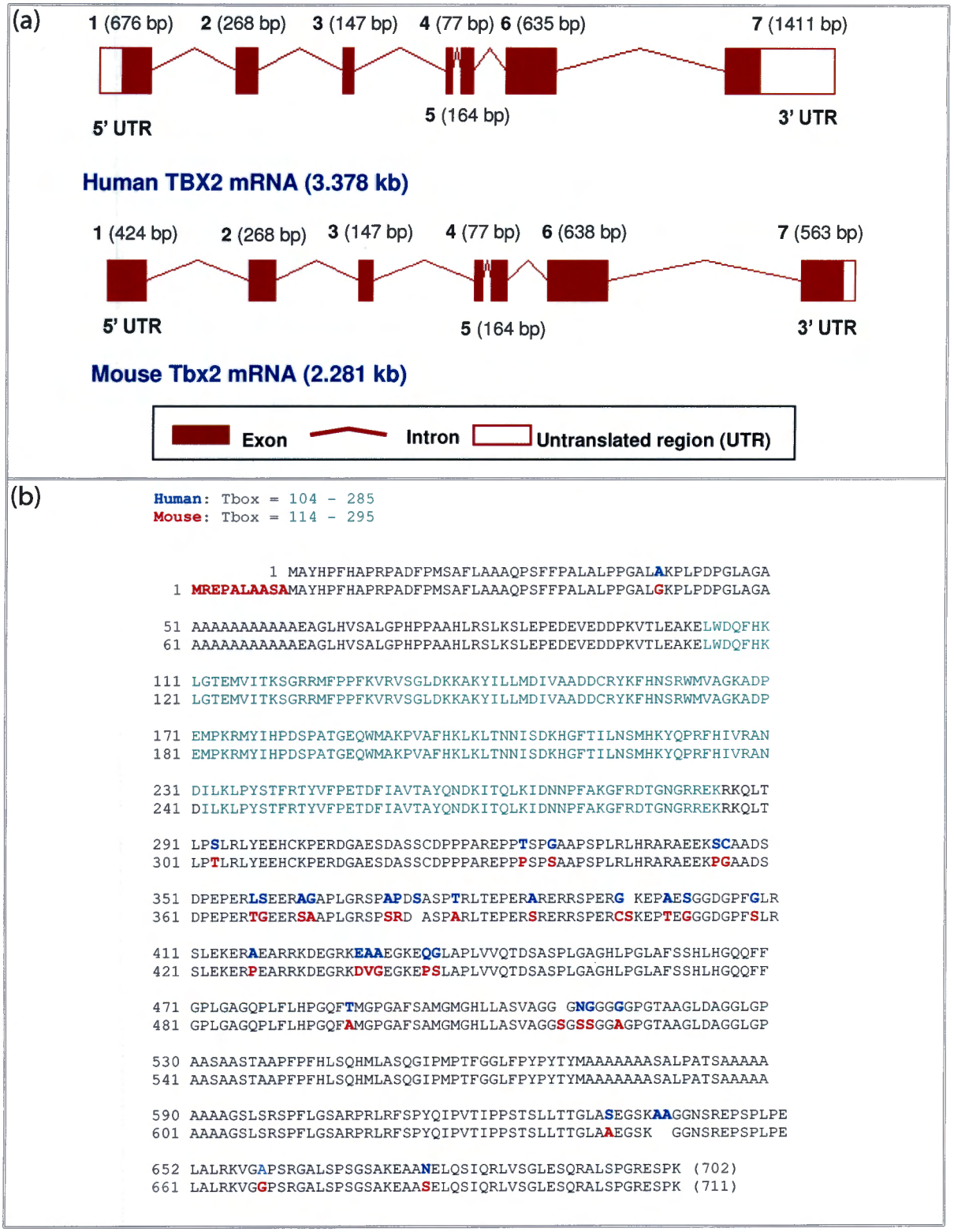


**Figure 1.1.** Comparative analysis of the Tbx2 gene and protein between human and mouse. (a) Schematic representation of the human and mouse Tbx2 gene depicting the relative positions and sizes of the 7 exons. (b) Alignment of the human and mouse Tbx2 protein. Conserved amino acid residues are in black font. The residues of the T-box important for DNA-binding are highlighted in green. Amino acid differences between human and mouse are indicated in blue and red, respectively. (From the ENSEMBL database, http://www.ensembl.org).

of severe cardiac defects (Harrelson et al., 2004). This result suggests a crucial role for Tbx2 during cardiac development.

In human the TBX2 protein consists of 702 amino acids with the T-box DNA-binding domain located at amino acids 104-285, whereas in mouse the T-box is located at amino acids 114-295 within the 711 amino acid protein (Fig. 1.1). Due to the high degree of homology between the DNA-binding domain of Brachyury and Tbx2, investigators have utilised Brachyury as a model to gain insight into the DNA-binding specificity of Tbx2. These studies show that Tbx2 binds as a monomer to the Brachyury single half-sites, recognising the consensus sequences GTGTGA, GGGTGA or GTGTTA (Carreira et al., 1998). Furthermore, TBX2 binds both the Brachyury palindromic and half sites equally well (Sinha et al., 2000). Mutating an arginine at position 122, which is conserved in all T-box factors, abolished the DNA-binding activity of TBX2 in vitro without affecting its stability and nuclear localisation (Sinha et al., 2000).

Unlike most members of the T-box family that function as transcription activators, Tbx2 is a potent repressor of its target genes (Carreira et al., 1998; He et al., 1999; Sinha et al., 2000). Using different Tbx2 deletion constructs linked to a CAT reporter gene containing a single T-element binding site, Paxton et al. (2002) was able to map the regulatory domains within the mouse Tbx2 protein. They identified two separate transcription repression domains; a novel amino-terminal repression domain (between amino acids 1-53) and confirmed the presence of a carboxy-terminal (between amino acids 529-573) repression domain identified in an earlier study (He et al., 1999; Paxton et al., 2002). A weak activation domain was also located within the T-box and depending on the promoter context; Tbx2 was capable of also activating gene expression (Paxton et al., 2002). Deletion of the carboxy-terminal repression domain only minimally reduces the ability of Tbx2 to repress gene expression, whereas deletion of both repression domains abolishes the repressor activity of Tbx2 (Paxton et al., 2002). This would suggest that the repression domain located in the amino-terminus is important in mediating the ability of Tbx2 to repress its target genes. The presence of both activation and repression domains within the Tbx2 protein suggests multiple but specific roles for this transcription factor depending on the cellular context or species. Indeed, in an attempt to identify genes that may be regulated by Tbx2, cDNA microarray analysis was performed on mouse NIH 3T3 fibroblasts overexpressing Tbx2 and the results revealed that the type 1 collagen gene was upregulated (Chen et al., 2001). Interestingly, a parallel investigation in which Tbx2 was overexpressed in the rat ROS17/2.8 osteoblastic cell line showed down-regulation of type I collagen (Chen et al., 2001). These contrasting results suggest that the cell context and/or the species may be important in determining the effect of Tbx2 on the expression of the type I collagen genes.

### 1.3.2 Role of Tbx2 in embryonic development

#### *Heart Development*

During cardiogenesis, Tbx2 is required for proliferation and patterning of the developing heart (Harrelson et al., 2004). The vertebrate heart originates from a linear tube that undergoes a series of complex looping and partitioning to form the multi-chambered organ. At the onset

of looping, the heart tube is compartmentalised into chamber myocardium, which form the atria and the ventricles, and non-chamber myocardium, which forms the outflow tract, the atrioventricular canal, the inflow tract and the inner curvatures. Together the chamber and non-chamber myocardium form the cardiac conduction system, which essentially controls the heart rate (reviewed in Christoffels et al., 2004). At around 8 and 10 days post coitus (dpc), *Tbx2* is detected in the non-chamber myocardium of the developing mouse heart (Fig. 1.2) (Christoffels et al., 2004; Habets et al., 2002). This is consistent with *Tbx2* expression patterns in chick heart development (Gibson-Brown et al., 1998; Yamada et al., 2000). *Tbx3* is co-expressed with *Tbx2* in the heart and it is postulated to function redundantly with Tbx2 in regulating target gene expression (Christoffels et al., 2004; Hoogars et al., 2004; Lingbeek et al., 2002). Bone morphogenic proteins (BMPs) belong to the TGF-β family of signalling transducers and are important in the development of the non-chamber myocardium (reviewed in Délot, 2003). There is accumulating evidence that *Tbx2* and *Tbx3* may be downstream Bmp2 targets during heart development. Firstly, the expression patterns of *Tbx2* and *Tbx3* overlap significantly with *bmp2* during chick heart development (Yamada et al., 2000). Secondly, ectopic expression of Bmp2 was shown to induce *Tbx2* and, to a lesser extent, *Tbx3* expression in non-cardiogenic tissue that is competent to form cardiac tissue. Moreover, in the same study when chick cardiac tissue explants were incubated with Noggin, a panantagonist of BMP signalling, *Tbx2* was downregulated with only very little effect on *Tbx3*. Furthermore, mouse *bmp2*$^{-/-}$ embryos show marked morphological abnormality in cardiac development (Zhang and Bradley, 1996) and *Tbx2* gene activity is substantially reduced in the cardiac region of these embryos (Yamada et al., 2000). *Tbx20*, which is required for the proliferation of myocardium (Cai et al., 2005), was also shown to be downregulated together with *Tbx2* and *Tbx3* in the developing hearts of Bmp mice in which the type 1 Bmp receptor was ablated (Yang et al., 2006). These studies indicate that the BMP pathway is an important upstream modulator of T-box gene expression during heart development.

Recent studies have identified Tbx2 target genes which have shed light on its functional role during early heart development. The formation of the chamber and non-chamber myocardium requires a distinct gene expression profile, and a number of chamber and non-chamber specific genes have been characterised. For example, expression of natriuretic precursor peptide type A (*Nppa*), connexin (*Cx*) *40*, *Cx43* and atrial natriuretic factor (*ANF*) are important for the differentiation and formation of the chamber myocardium (Christoffels et al., 2000; Christoffels et al., 2004). Formation of the non-chamber myocardium on the other hand requires the expression of a different set of genes which involves *Tbx2* (Christoffels et al., 2004; Habets et al., 2002). Tbx2 normally inhibits cell proliferation and chamber differentiation in non-chamber myocardium, and it is thought that chamber differentiation requires its repression by Tbx20 (Cai et al., 2005). Tbx20, a positive regulator of chamber formation, binds and transcriptionally represses *Tbx2* via a T-element (Cai et al., 2005). Given the ability of Tbx2 to repress *Nmyc1*, which is required for the normal proliferation of the heart (Davis and Bradley, 1993), it is proposed that in chamber myocardium Tbx20 represses *Tbx2* thus preventing its repression of *Nmyc1* (Cai et al., 2005). However, in non-chamber myocardium repression of *Tbx2* by Tbx20 is abolished, allowing Tbx2 to repress *Nmyc1* and the early cardiac genes, resulting in decreased

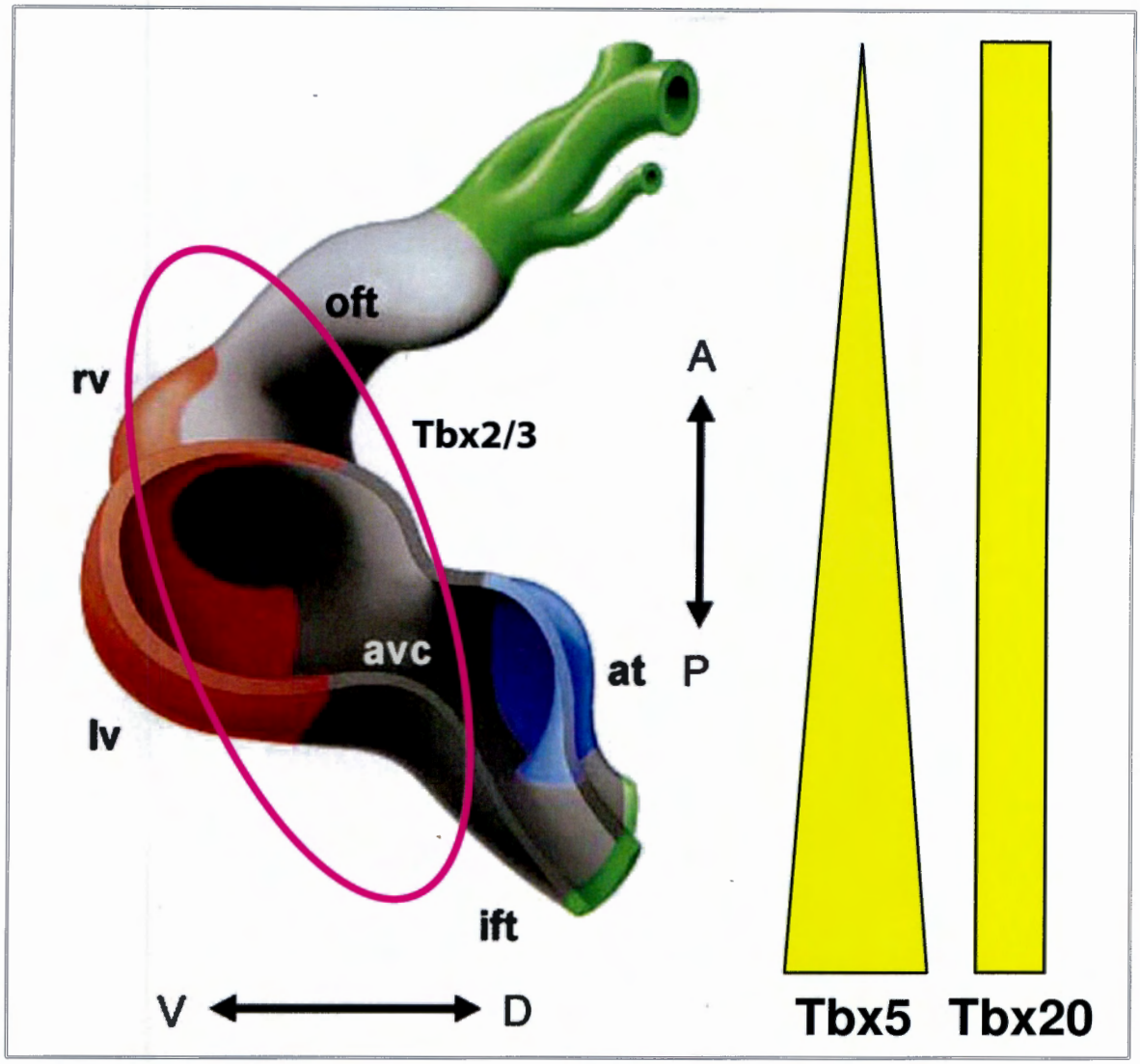


**Figure 1.2.** The role of Tbx2, Tbx3, Tbx5 and Tbx20 in early heart development. Schematic representation of an E9.5–10.5 chamber forming heart showing T-box patterning in the different emerging structures. Tbx2 and Tbx3 exert their function in the non-chamber myocardium, Yellow bars indicate expression patterns of Tbx5 and Tbx20. Tbx5 is required for anterior-posterior patterning and, along with Tbx20, for chamber differentiation. The primary myocardium is in gray, the ventricular myocardium is in red, and the atrial myocardium is in blue. A, anterior; at, atrium; avc, atrioventricular canal; D, dorsal; ift, inflow tract; lv, left ventricle; oft, outflow tract; P, posterior; rv, right ventricle; V, ventral. (Modified from Christoffels et al., 2004 and Hoogaars et al., 2007).

proliferation within this region (Cai et al., 2005; Stennard et al., 2005). In vitro reporter assays and transgenic mice studies have shown that during non-chamber formation, Tbx2 represses the transcription of *Nppa*, *Cx40, Cx43* and *ANF* and thus plays a role regulating the formation of the multi-chambered heart (Christoffels et al., 2004; Habets et al., 2002).

During heart development the non-chamber myocardium retains the embryonic myocardial phenotype of the tubular heart longer than the chamber myocardium. The *ANF* gene is specifically expressed in the developing chamber myocardium and is one of the first hallmarks of chamber formation (Christoffels et al., 2000), which raises the question of how this gene is repressed in the non-chamber myocardium. This was addressed by studies which found that mutating two adjacent binding sites for T-box factors and Nkx2.5, respectively, abrogated repression of *ANF* in regions of the non-chamber myocardium (Habets et al., 2002). Furthermore, whereas Tbx2 and Tbx3 were shown to co-operate with Nkx2.5 to suppress *ANF* promoter activity in embryonic myocardium (Habets et al., 2002; Hoogaars et al., 2004), Tbx5 conversely bound the same site and co-operated with Nkx2.5 to activate *ANF* gene expression in chamber myocardium (Bruneau et al., 2001; Hiroi et al., 2001). Together, these findings point to a complicated network in which several T-box factors act in the patterning, proliferation and morphogenesis of the developing heart.

### *Brain Development*

The hypothalamus is situated in the area of the brain just below the thalamus and is important in regulating body temperature, blood pressure, weight regulation, and many other autonomic-nervous system activities such as fear, rage and sexual behaviors (van de Graaff, 2002). Both in vitro and in vivo studies have implicated Tbx2 in the patterning and development of the hypothalamus. Much of the evidence for this results from work done in chick on a subset of hypothalamic cells, termed the ventral tubero-mamillary (vt-m) cells which arise from a set of floor plate-like precursors (Manning et al., 2006). The floor plate-like precursors initially express *Sonic hedgehog* (*Shh*) but require its downregulation in order to progress to vt-m cells. BMPs have previously been shown to downregulate *Shh* in a wide variety of tissues and Manning et al. (2006) show that downregulation of *Shh* in the vt-m cells occurs by a BMP-Tbx2 signalling pathway. Both overexpression and knock-down experiments showed that Tbx2 downregulates *Shh* in the vt-m hypothalamus. Furthermore, Jeong and Epstein (2003) identified a T-element in the mouse *Shh* promoter and they show that mutating this T-element result in aberrant expression of *Shh* within the hypothalamus. In Manning et al. (2006), BMPs were shown to upregulate *Tbx2*, which may involve the ability of BMPs to antagonise Wnt. In the presence of the BMP inhibitor, chordin, expression of *Tbx2* was downregulated in the vt-m hypothalamus. In contrast, blocking BMP and Wnt signalling was shown to rescue the expression of *Tbx2* in the vt-m cells. The results by Manning et al. (2006) raise the possibility that the T-element in *Shh* is regulated by Tbx2 in the hypothalamus.

### *Eye Development*

Expression of Tbx2 and other members of the T-box family may be important for the growth and patterning of the mammalian embryonic eye. In the mouse embryo, eye development begins

at embryonic day 8.5 and involves the formation of a bilayered optic cup which gives rise to the neural retina (inner layer) and the retinal pigmented epithelium (outer layer) (reviewed in Chow and Lang, 2001). In human and mouse *TBX2/Tbx2*, *TBX3/Tbx3* and *TBX5/Tbx5* are expressed asymmetrically across the neural retina with high levels of the transcripts detected in the dorsal and peripheral regions (Sowden et al., 2001). The BMP4 signalling pathway plays an important role in the patterning of the optic cup, where its expression pattern in the dorsal neural retina overlaps with that of the Tbx2 subfamily members, *Tbx2*, *Tbx3* and *Tbx5* (Behesti et al., 2006). To test whether BMP4 signalling is able to regulate the expression of these T-box genes during eye development, a combination of whole mouse embryo cultures and bead implantation experiments were performed. In this study, beads soaked in BMP4 or the BMP antagonist, Noggin, were implanted into whole mouse embryos across the dorsal-ventral axis of the optic cup. Exogenous BMP4 protein resulted in the expansion of the zone of *Tbx2* and *Tbx3* expression into the ventral retina, while the expression pattern of *Tbx5* remained restricted to the dorsal retina. However, while Noggin abolished *Tbx5* expression it only reduced expression of *Tbx2* while having no affect on *Tbx3*. The response of *Tbx2*, *Tbx3* and *Tbx5* to BMP4 signalling was therefore different for each T-box gene, suggesting that the effect of BMP4 signalling varies across the optic cup. Furthermore, increasing the level of BMP4 signalling not only resulted in a decrease in proliferation but also in alterations in the growth and shape of the eye. Considering the proliferative role previously identified for Tbx2 and Tbx3 during development it is tempting to suggest that as downstream targets of BMP4 they may also be important in regulating the proliferation and development of the eye (Behesti et al., 2006).

***Bone Development***

Tbx2 has been shown to regulate expression of the genes encoding *connexin* (*Cx*) *43* (as mentioned earlier in heart development) and collagen, which are factors involved in bone formation. Gap junctions, composed of connexin (Cx) protein subunits, function to connect the cytoplasm of adjacent bone (osteocytes) cells and are therefore important in cell-cell communication. Cx43 is the predominant gap junction protein in bone and its regulated temporal pattern of expression has been shown to play a critical role in normal ossification and osteoblast function (Lecanda et al., 2000). *Tbx2* and *Cx43* are expressed in osteogenic progenitors and osteoblasts, which suggested that Cx43 may be a potential Tbx2 target gene. This was tested in a study in which a rat osteosarcoma cell line was transfected with either sense or anti-sense *Tbx2* and the results showed that inhibition of *Tbx2* resulted in a marked increase in *Cx43* expression (Borke et al., 2003; Chen et al., 2004). The *Cx43* promoter has several T-element half sites and Tbx2 was shown to directly bind and repress the *Cx43* promoter at two of these sites (Chen et al., 2004). Interestingly, in the same study transgenic mice injected with the LacZ reporter cloned downstream of either the wild-type (WT) or mutant *Cx43* promoter in which the two T-elements were mutated exhibited the same expression pattern of LacZ (Chen et al., 2004). These results suggest that in vivo, Tbx2 may require other factors to regulate *Cx43* expression. It is also important to note that these studies were done in mouse and rat, suggesting that the role of Tbx2 during bone development may vary between species. Recent studies have implicated the mouse Type 1 collagen gene as a potential Tbx2 target (Chen et al., 2001).

Type 1 collagen synthesis is crucial for normal embryonic development and in maintaining tissue integrity, and its aberrant expression has deleterious effects on several biological processes including bone development (reviewed in Bornstein and Sage, 1989). In an attempt to identify genes that may be regulated by Tbx2, cDNA microarray analysis was performed on mouse NIH 3T3 fibroblasts overexpressing Tbx2 and results revealed that the type 1 collagen gene was upregulated (Chen et al., 2001). Interestingly, a parallel investigation in which Tbx2 was overexpressed in the rat ROS17/2.8 osteoblastic cell line showed downregulation of type 1 collagen (Chen et al., 2001). A recent study in our laboratory showed that TBX2 was able to repress α2(1) collagen expression independent of a T-element, suggesting that TBX2 may be acting as a co-repressor in these studies (Teng et al., 2007). Taken together, these contrasting results suggest that Tbx2 may regulate type 1 collagen genes by functioning as either co-activator or co-repressor depending on the cell context and/or the species.

### *Limb and Digit Development*

The development of the vertebrate limb begins with the formation of the limb primordium (limb bud) which is strategically positioned in the embryo, enabling it to receive and interpret a tightly regulated network of factors which provide positional cues. The limb bud, composed of mesenchymal cells, is surrounded by an ectodermal layer which extends out of the body wall. Interactions between the epithelial and mesochymal cell layers govern the growth and patterning of the limb bud (reviewed in Capdevila and Izpisúa Belmonte, 2001). The mature limb is a complex structure composed of many tissue types such as bone, muscle and tendon (reviewed in Logan, 2003). Many genes required for limb development are expressed in identical patterns in both the developing forelimb and hindlimb, which raises the question of which genes are required for specifying limb-type identity. Recent studies have begun to explain how genes that specify limb-type identity interact with signalling cascades common to both forelimb and hindlimb.

*Tbx2*, *Tbx3*, *Tbx4* and *Tbx5* are expressed in developing mouse limbs either at the time of limb field specification, during limb bud outgrowth, or both (Chapman et al., 1996; Gibson-Brown et al., 1996). The temporal and spatial expression patterns of *Tbx4* and *Tbx5* are primarily restricted to the developing hindlimb and forelimb, respectively (Gibson-Brown et al., 1996; reviewed in Ruvinsky and Gibson-Brown, 2000), which is consistent with their having roles in specifying limb-type identity. The roles of *Tbx4* and *Tbx5* seem to be tightly linked to the activity of the signalling proteins FGF, BMP and Wnt which are required for limb outgrowth and patterning (Rodriguez-Esteban et al., 1999).

The spatial and temporal expression of *Tbx2* and *Tbx3* along the anterior and posterior margins of the fore-and hindlimb buds is generally very similar in mouse and chick (Chapman et al., 1996; Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998). A study by Suzuki et al. (2004) shows that Tbx2 and Tbx3 play an important role in the determination of digit identity in chick. *Tbx3* was found to be important in the specification of digit III, while both *Tbx2* and *Tbx3* were required for digit III and IV identity. Furthermore, in this study the investigators showed that dominant negative forms of *Tbx2* and *Tbx3* lead to

reduced levels of *Shh* and that conversely, misexpression of *Tbx2* induced anterior expansion of *Shh* expression. It is important to note that normal patterning of the developing limb requires the confinement of *Shh* expression to the posterior margin of the limb bud and thus the study by Suzuki et al. (2004) would suggest that Tbx2 and Tbx3 play an important role as a positive regulator of *Shh* expression within the posterior limb bud. In a very recent study, Nissim et al. (2007) also make a very strong case in favour of Tbx2 being responsible for limiting *Shh* expression to the margins of the limb bud. In the presence of cyclopamine, a Shh inhibitor, the expression of *Tbx2* was unaffected in the anterior and posterior margins of the limb bud. In the same study, the investigators identified a novel ectodermal signalling centre, which was found to be sufficient to induce and maintain the expression of *Tbx2* in the underlying mesoderm of the limb. Furthermore, they show that grafting the ectodermal signalling centre from the anterior margin to the dorsal region of another limb induced the anterior expansion of *Shh*. These results suggest that signals from the ectodermal signalling centre induce expression of Tbx2 which regulates *Shh* expression in the posterior margin of the limb. Taken together, this is in keeping with the observation that in the human ulnar-mammary syndrome, haploinsufficiency of *TBX3* leads to limb defects, which corresponds with that seen when *Shh* expression is reduced or absent (Bamshad et al., 1997; Davenport et al., 2003). These experiments beg the question of how Tbx2 and Tbx3 are regulated during limb development, which would be an important direction for future studies.

***Mammary gland Development***

Both Tbx2 and Tbx3 have been implicated in the development of the mammary glands in mice and humans (reviewed in Rowley et al., 2004). Both factors are expressed in the developing mammary glands of mice, where they are expressed in specific spatiotemporal patterns in the mesodermal and epithelial cell layers (Chapman et al., 1996; reviewed in Rowley et al., 2004). During the emergence of the mouse mammary placodes, *Tbx2* is expressed in the underlying mesodermal cells, while *Tbx3* is expressed only in the epithelial cells. Interactions between the epithelial and mesodermal cell layers are known to govern induction and development of the mammary glands, suggesting a role for Tbx2 and Tbx3 in the induction of the mammary placodes and specification of mammary identity (reviewed in Imagawa et al., 2002 and Hennighausen and Robinson, 2001). The importance of Tbx3 during mammary development is demonstrated by the fact that homozygote mice fail to induce formation of the mammary glands (Davenport et al., 2003) while heterozygotes exhibit hyoplasia and nipple abnormalities (Bamshad et al., 1997, 1999). Furthermore, individuals suffering from ulnar-mammary syndrome are prone to breast abnormalities which include absent areola, inability to lactate and mild hypoplasia of the breast. It is important to note that the expression of *Tbx2* and *Tbx3* in mammary gland tissue persists into adulthood (reviewed in Rowley et al., 2004). This implicates them in the entire process of mammary gland development and renewal as these glands are constantly undergoing changes depending on a female's state of puberty, pregnancy, lactation or involution. Compared to Tbx3, the role of Tbx2 in the development of the mammary glands is less well defined. However, the defects in *Tbx3* heterozygous mice are exacerbated in *Tbx2*, *Tbx3* double heterozygote mice which not only suggest a role for Tbx2 in mammary gland development but also imply a genetic interaction between these two factors (Jerome-Majewska et al., 2005).

***Melanocytes***

*Tbx2* expression has been observed in several melanocyte and melanoma cell lines but not in pre-melanoblast cells (Carreira et al., 1998). Melanoblasts are neural crest derived cells which migrate to the hair follicles and epidermis where they differentiate into mature melanocytes capable of producing pigment (Silvers, 1979). The microphthalmia associated transcription factor, MITF, a member of the helix-loop-helix leucine zipper class, has been identified as a key regulator of melanocyte development (Hodgkinson et al., 1993; reviewed in Levy et al., 2006). MITF was shown to be involved in melanocyte differentiation, proliferation and survival. To date, the MITF target genes identified encode proteins involved in pigmentation including tyrosinase and tyrosinase-related protein 1 (TRP-1) (Bentley et al., 1994; Ganss et al., 1994; Hemesath et al., 1994; Yasumoto et al., 1994; Yasumoto et al., 1995; Yavuzer et al., 1995). Interestingly, MITF was found to bind and activate the expression of *Tbx2* which raises the question of what the role of Tbx2 is in melanocytes (Carreira et al., 2000). Since Tbx2 is not involved in melanin production one might expect that as a MITF target it may be involved in the other functions of MITF such as melanocyte proliferation and/or survival. The fact that Tbx2 is expressed after the cells have committed to the melanocytes lineage (Carreira et al., 1998) also suggests that Tbx2 may play a role in the maintenance of melanocyte identity.

### 1.3.3 Role of Tbx2 in the regulation of the cell cycle and cancer

There is a significant amount of evidence pointing to a role for Tbx2 and Tbx3 in cell cycle regulation and cancer. Both TBX2 and TBX3 gene expression have been found to be de-regulated in a number of cancers, including breast (Sinclair et al., 2002; Fan et al., 2004), pancreatic (Mahlamäki et al., 2002; Hansel et al., 2004), melanoma (Vance et al., 2005; Hoek et al., 2004), liver (Renard et al., 2007) and bladder (Ito et al., 2005) cancers.

TBX2 and TBX3, as mentioned earlier, are involved in normal breast development and studies have shown that their altered expression may play a role in the pathogenesis of breast cancer. *TBX2* was shown to be amplified and overexpressed in a subset of breast cancer cell lines and primary tumours. Using fluorescence *in situ* hybridization, *TBX2* was shown to be amplified in BRCA1- and BRCA2-related breast tumours (Jacobs et al., 2000; Sinclair et al., 2002). Furthermore, *in situ* hybridization of paraffin sections from sporadic and hereditary breast tumours confirmed that this amplification was a result of increased *TBX2* gene expression (Sinclair et al., 2002). Similarly, *TBX3* is amplified in a subset of breast cancer cell lines (Fan et al., 2004) and was shown compared to non-cancer patients to be upregulated in plasma from breast and ovarian cancer patients (Lomnytska et al., 2006). Both *TBX2* and *TBX3* have also been associated with pancreatic cancer. While *TBX2* was found to be overexpressed in 50% of thirty one pancreatic cancer cell lines tested (Mahlamäki et al., 2002), *TBX3* was shown to be upregulated in metastatic pancreatic endocrine neoplasms (Hansel et al., 2004). Importantly, TBX2 maps to chromosomal band 17q23 (Law et al., 1995), which is frequently amplified in many breast and pancreatic cancer cells (Wu et al., 2001, Sinclair et al., 2002; Bärlund et al., 2000; Mahlamäki et al., 2002). Furthermore, both TBX2/Tbx2 and TBX3 are expressed in normal melanocytes and have been found to be strongly upregulated in a subset of melanoma cell lines (Carreira et al., 1998; Vance et al., 2005; Hoek et al., 2004). Dosage sensitivity of

TBX2 and TBX3 has also been associated with other cancers. TBX2, which is expressed in normal human fibroblasts, was recently shown to be downregulated in several transformed fibroblast cell lines (Teng et al., 2007), while TBX3 was found to be upregulated in transformed lung fibroblast cells (Yin et al., 2004). Although the above findings suggest an association of both TBX2 and TBX3 with tumourigenesis, they do not provide a mechanism(s) for how these T-box genes contribute to the oncogenic process.

A possible mechanism for how TBX2 and Tbx3 may contribute toward the oncogenic process is suggested by studies which have shown that they are both able to function as immortalising genes that enable the cells to bypass senescence (Jacobs et al., 2000; Carlson et al., 2001). Cell senescence is broadly defined as the physiological program of irreversible growth arrest which can be triggered by shortening of the telomeres (replicative senescence) or by different forms of stress (accelerated senescence) (reviewed in Roninson, 2003). The main mediators of senescence are the cyclin dependent kinase inhibitors (Cdkis), p21 and $p16^{INK4a}$, with p21 thought to be necessary for initiating the senescence-like growth arrest and $p16^{INK4a}$ required for maintenance of the state (Stein et al., 1999). The induction of p21 appears to be largely p53-dependent and the Cdki, $p19^{ARF}$, has also been shown to initiate a senescent-like growth arrest by stabilising p53 protein levels (reviewed in Roninson, 2003). Senescence is thought to be an important protective mechanism against cancer as neoplastic transformation appears to result from events that inhibit the program of senescence. Using two different cell culture models of senescence, investigators have shown that both Tbx2 and Tbx3 can behave as immortalising genes by preventing senescence. Primary mouse embryonic fibroblasts (MEFs) deficient for the proto-oncogene *Bmi1* ordinarily undergo premature senescence which is bypassed when they are infected with retroviral vectors expressing either TBX2 or TBX3 (Jacobs et al., 2000; Brummelkamp et al., 2002). Similar results were observed using a mouse striatum cell line (ST. $Hdh^{q111}$) expressing a temperature-sensitive SV40 T-antigen which allows them to proliferate at 33°C but to senesce at 39°C. In these experiments, when the ST.$Hdh^{q111}$ cells were infected with either a Tbx2 or TBX3-expressing retrovirus they fail to undergo senescence at 39°C (Brummelkamp et al., 2002; Prince et al., 2004). In the above studies TBX2 and TBX3 were shown to prevent senescence by a mechanism involving their ability to transcriptionally repress $p19^{ARF}$ (mouse homologue of the human $p14^{ARF}$) and p21 (Jacobs et al., 2000; Brummelkamp et al., 2002; Lingbeek et al., 2002; Prince et al., 2004). The possibility that Tbx2 may contribute to the oncogenic process by inhibiting senescence is supported by a study that showed that expressing a dominant-negative Tbx2 in B16 mouse melanoma cells led to increased levels of p21 and induction of senescence (Vance et al., 2005).

Another possible mechanism by which Tbx3 may contribute to oncogenesis is through inhibiting the $p19^{ARF}$/Mdm2/p53 pathway. In response to abnormal mitogenic signalling, activation of oncogenes such as Myc and Ras stimulate the $p19^{ARF}$/Mdm2/p53 pathway. Briefly, aberrant mitogenic signalling leads to increased $p19^{ARF}$ levels which results in $p19^{ARF}$ sequestering the ubiquitin ligase murine double minute (Mdm2), thus preventing ubiquitin-mediated degradation of the p53 protein. Once activated, p53 transcriptionally regulates a set of genes involved in cell cycle arrest, DNA repair or apoptosis (Zindy et al., 1998; Palmero et al., 1998; reviewed in Sherr

and Weber, 2000). Interestingly, Tbx3 was found to cooperate with oncogenic Myc and Ras to efficiently transform MEFs, which correlated with an ability of Tbx3 to suppress the induction of $p19^{ARF}$ and p53 protein levels (Carlson et al., 2002). In the same study, ectopic Tbx3 expression was shown to protect MEFs against Myc-induced apoptosis. Additional support for the ability of Tbx3 to block apoptosis was demonstrated when Tbx3 was silenced in rat bladder carcinoma cells and apoptosis was induced (Ito et al., 2005). Furthermore, Renard et al. (2007) showed both in vitro and in vivo that human liver tumour cells stably transfected with dominant negative TBX3 exhibited severely reduced proliferation and tumour growth. Moreover, silencing TBX3 using siRNA inhibited anchorage independence and failed to protect cells against apoptosis.

Taken together all the above information suggests that Tbx2 and Tbx3 may play an important role in cell cycle regulation. If this were the case then one might expect the protein levels of Tbx2 and Tbx3 to be regulated during the various phases of the cell cycle. Indeed, Bilican and Goding (2006) showed that Tbx2 protein levels are regulated during the cell cycle, with levels peaking at G2 and the protein being almost undetectable in mitosis. The observation that Tbx2 protein levels peak at G2 points to a possible role for Tbx2 during the G2/M transition of the cell cycle. Indeed, a recent investigation in our laboratory has provided evidence for a functional role for TBX2 during G2/M (Davis et al., 2007). Ectopic expression of TBX2/Tbx2 in a fibroblast and a melanoma cell line was shown to lead to a number of mitotic defects. FACS analysis of the TBX2/Tbx2 expressing cells showed they are initially arrested in G2/M but then acquire the ability to override the block and re-enter the cell cycle with a DNA content of 8n. Compared to the parental cells the TBX2/Tbx2 expressing cells displayed increased features of genomic instability, such as chromosome doubling, chromosomal rearrangements and polyploidy. The ability of TBX2/Tbx2 to override the checkpoint controls resulting in genomic instability may provide an additional mechanism by which TBX2/Tbx2 contributes to tumourigenesis. Furthermore, the studies by Bilican and Goding (2006) and Davis et al. (2007) both highlight the importance of the need for TBX2/Tbx2 protein levels to be tightly regulated during the cell cycle. Interestingly, the study by Bilican and Goding (2006) also shows that changes in Tbx2 protein levels are not matched by changes in *Tbx2* mRNA levels, suggesting that the protein may be regulated by post-translational modifications such as phosphorylation. A major aspect of the current thesis was to investigate whether the Tbx2 protein is indeed phosphorylated and regulated by the cyclin dependent kinases and hence the next section will provide an overview of the key areas relevant to the research presented.

## 1.4 The eukaryotic cell cycle

The eukaryotic cell cycle, which is divided into the G1, S, G2 and M phases, represents a series of tightly regulated events that allows the cell to duplicate its genetic material and divide into two identical daughter cells. The major events that need to be regulated are DNA synthesis (S phase) and the equal separation of the duplicated chromosomes between two daughter cells (M or mitosis) (Fig. 1.3) (reviewed in Tessema et al., 2004). G1 and G2 are the checkpoints that control the order and timing of cell cycle transitions and ensure that the events of S and M are completed with high fidelity. In G1, the cell size is monitored, while in G2 the replicated chromosomes and cellular structures are checked before proceeding into mitosis. In addition

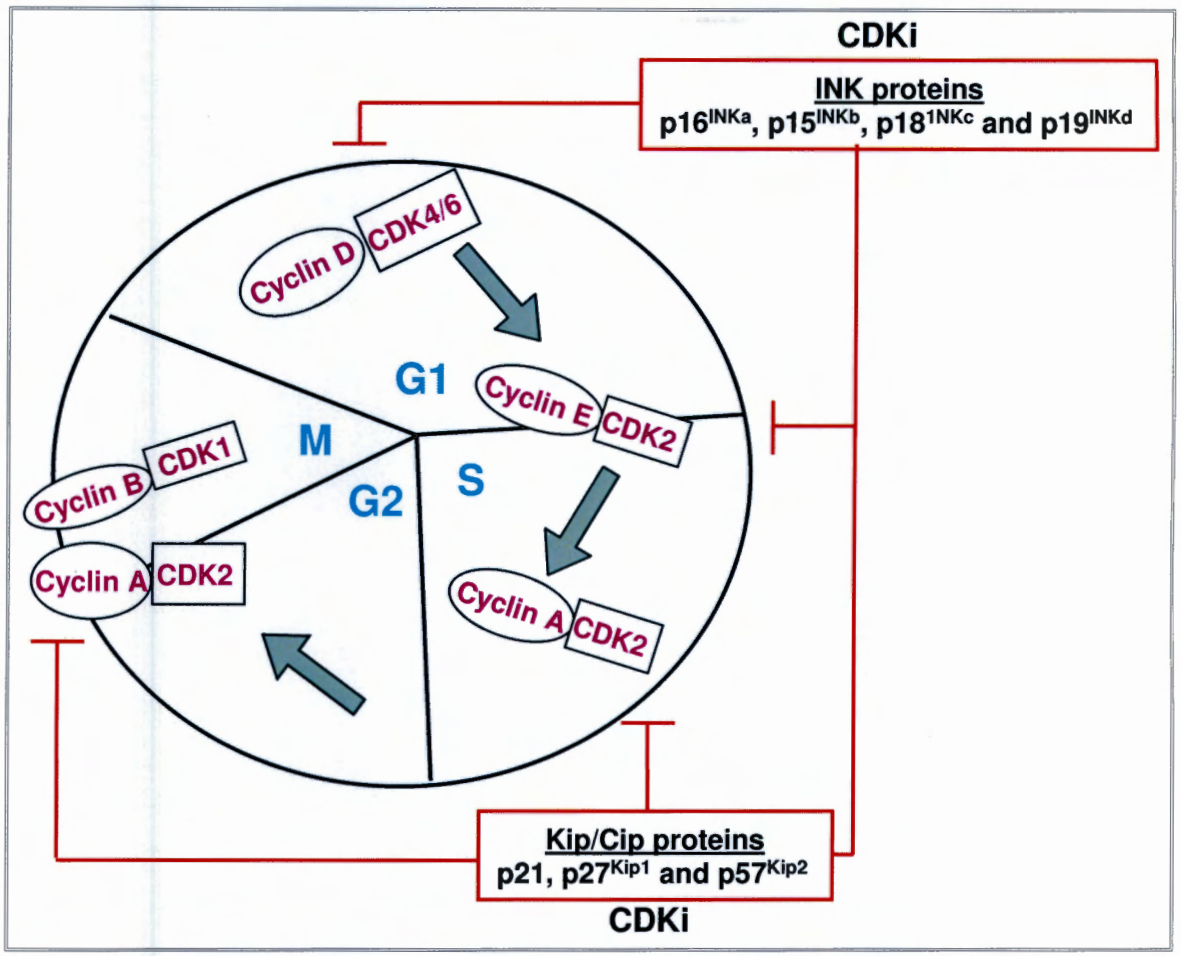


**Figure 1.3.** The eukaryotic cell cycle. The cell cycle is divided into four phases (G1, S, G2 and M). Progression through the different phases of the cell cycle is mediated by cyclin dependent kinases (Cdk), which are positively regulated by cyclins and negatively regulated by cyclin dependent kinase inhibitors (Cdki).

to G1 and G2 there is also the spindle assembly checkpoint (SAC) in mitosis that monitors the successful completion of the metaphase-to-anaphase transition. SAC ensures that the mitotic chromosomes are correctly aligned on the mitotic spindle and that the sister chromatids are properly separated to opposite ends of the poles. All three checkpoints are also able to respond to DNA damage and if triggered will arrest the cell in order to for example, allow the cell time to repair the DNA. Checkpoint loss results in genomic instability which is an important hallmark of several cancers.

### 1.4.1 Cyclins, cyclin dependent kinases (Cdks) and cdk inhibitors (Cdkis)

Progression through the different phases of the cell cycle is mediated by two classes of protein, the cyclins and Cdks (reviewed in Pines, 1995 and Morgan, 1997). Cdks are serine/threonine kinases that are activated during specific stages of the cell cycle and are often referred to as the engines that drive the cell cycle. In mammals, 11 Cdks have been identified, although only five (Cdk1, Cdk2, Cdk3, Cdk4 and Cdk6) have been directly implicated in the regulation of the cell cycle (reviewed in Malumbres and Barbacid, 2005). Activation of Cdks is dependent on binding to a regulatory subunit called cyclins which, as the name suggests, are proteins characterised by their tightly regulated appearance and disappearance during the cell cycle, which are controlled by precise transcription and proteolytic events. Degradation of the cyclins involves a sequence within their N-termini called the destruction box (D-box) and studies have shown that deletion or introduction of point mutations within the D-box inhibits their proteolytic degradation (Glotzer et al., 1991; Yamano et al., 1998; Geley et al., 2001). The destruction of the cyclins occurs by ubiquitination within the D-box, which targets them for proteolytic degradation (reviewed in Fung and Poon, 2005).

Association of Cdks with their appropriate cyclins regulates progression through the phases of the cell cycle (Fig. 1.3) by phosphorylating substrates required for the events of specific phases. For example, progression through G1 is regulated by cyclin D/Cdk4/6, G1/S transition by cyclin E/Cdk2, S phase by cyclin A/Cdk2 and entry into M requires both cyclin A/Cdk2 and cyclin B/Cdk1 (reviewed in Morgan, 1997 and Schwartz and Shah, 2005). In the absence of appropriate mitogenic stimuli or in response to DNA damage, the cell is arrested at any one of the checkpoints mentioned above by the Cdk inhibitors (Cdkis) (reviewed in Elledge, 1996 and Sherr and Roberts, 1999). Two families of Cdkis have been identified, namely the Kip/Cip proteins (p21, $p27^{Kip1}$ and $p57^{Kip2}$) and the INK proteins ($p16^{INKa}$, $p15^{INKb}$, $p18^{INKc}$ and $p19^{INKd}$). The Kip/Cip family of inhibitors form a ternary structure with cyclin/Cdk complexes containing cyclins D, E and A, while the INK family prevent assembly of the Cdk with the cyclin and selectively binds cyclin D/Cdk4 or 6. The kinase activity of Cdks is therefore regulated by Cdkis, together with specific phosphorylation and dephosphorylation events, while the cyclins are tightly regulated both transcriptionally and by ubiquitin-dependent degradation.

As mentioned earlier in 1.3.3, Tbx2 levels peak at G2 and it appears to have a role in either G2 and/or the transition from G2 to M. If this is the case it would be expected to be a substrate for the cyclin/CDK complexes that regulate these phases. The next section will therefore deal specifically with the cyclin A/Cdk2 and cyclin B/Cdk1 complexes.

### 1.4.2 Regulation of the transition from G2 to M by cyclin A/Cdk2 and cyclin B/ Cdk1

The production of two identical daughter cells during mitosis occurs in five phases: prophase, prometaphase, metaphase, anaphase and telophase (reviewed in Pines and Rieder, 2001 and Ferrari, 2006). At the end of S phase when DNA has been replicated, sister chromatids are held together by kinetochores at their centromeric regions and by cohesins along their entire length. Very briefly, chromatin condensation, which coincides with histone H3 phosphorylation, occurs during prophase. Nuclear envelope breakdown which occurs at the time of transition to prometaphase is facilitated by phosphorylation of the nuclear lamins. At the same time spindle microtubules make contact with kinetochores at the chromatids. As the cell approaches metaphase, the chromosomes begin to move to the equator of the spindle apparatus, where they align along the metaphase plate. During anaphase the sister chromatids move to opposite poles of the spindle by the shortening of the microtubules that are attached to kinetochores. Mitosis is concluded at telophase with the reformation of the nuclear membrane around decondensing chromosomes. This is followed by the cleavage and separation of the two daughter cells during cytokinesis.

Cyclins A and B are regarded as the mitotic cyclins as they are important in regulating the entry into and exit from mitosis. In mammalian cells, there are two types of A cyclins, namely A1 and A2 and three types of B cyclins (B1, B2 and B3) (reviewed in Fung and Poon et al., 2005). Cyclin A1 is only expressed during meiosis, while cyclin A2 is expressed in all proliferating somatic cells. Of the three B-type cyclins, only cyclin B1 is associated with mitosis. Cyclin B3 is restricted to the developing germ line cells and the adult testis, and while cyclin B2 is co-expressed with cyclin B1 it is localised to the Golgi apparatus and does not seem to have a recognised role as yet.

It is well established that cyclin B1 protein forms a complex with Cdk1 which, when activated, initiates the entry, transition and exit from mitosis by phosphorylating numerous substrates (reviewed in Nigg, 2001 and Ferrari et al., 2006). These include proteins that control chromosome condensation such as condensins, nuclear lamins (the phosphorylation of which leads to nuclear envelope breakdown), microtubule binding proteins and kinesin-related motor proteins, which are involved in centrosome separation and spindle assembly. Exit from mitosis is regulated by anaphase-promoting complexes/cyclosome (APC/C) which targets cyclin B1 and mitotic kinases such as securin, aurora-A and –B among others for proteolytic degradation (Sudakin et al., 1995; Zachariae et al., 1998; reviewed in Harper et al., 2002 and Peters, 2002). A major requirement for transition from metaphase to anaphase is the degradation of cyclin B1 through the activity of APC/C which, interestingly, is a target of cyclin B1/Cdk1 (reviewed in Fung and Poon, 2005).

As mentioned earlier, cyclin A/Cdk2 has a dual role in the regulation of the S and M phases of the cell cycle. During mitosis, cyclin A can also associate with both Cdk1 and Cdk2 but the cyclin A/Cdk2 complex is the most active and abundant of the two complexes (Pagano et al., 1992, 1993; Rosenblatt et al., 1992). Once active, cyclin A/Cdk2 is thought to promote entry into

mitosis by promoting the activation of cyclin B1/Cdk1 (Guadagno and Newport, 1996; Lukas et al., 1999). This possibility was supported by results from a study by Gong et al. (2007) which showed that when cyclin A was knocked down by RNAi, nuclear envelope breakdown did not occur and that cyclin B1 did not translocate to the nucleus. Furthermore, cyclin A/CDK2 is also thought to be required for progression of cells up to mid-prophase because it was shown that once cells have replicated their DNA, cyclin A/CDK2 promotes entry into mitosis and initiates chromatin condensation independent of cyclin B1/CDK1 (Furuno et al., 1999).

### 1.4.3 Regulation of cyclin A/Cdk2 and cyclin B/Cdk1

The activity of the cyclin A/Cdk2 and cyclin B/Cdk1 complexes is tightly controlled by the levels of the cyclin A and B1 regulatory subunits, as well as by subcellular localisation and specific phosphorylation events on the Cdk1 and Cdk2 catalytic subunits.

#### *Levels of cyclin A and B1*

Cyclin A starts to accumulate during G1 with levels peaking in G2 and the protein is degraded at metaphase (den Elzen and Pines, 2001; Geley et al., 2001; Pines and Hunter, 1990, 1991; Clute and Pines, 1999). Cyclin B1 protein accumulates at G2 and is degraded slightly later than cyclin A at the metaphase-anaphase transition. Both cyclin A and B1 possess an N-terminal region called the D-box which targets them to the APC/C complex for ubiquitination (reviewed in Fung and Poon, 2005). Despite the fact that cyclin A and B1 are degraded by the same mechanism it is not clear why these two cyclins are degraded at slightly different times. It has however been proposed that variations in the D-box may account for the difference in the destruction of the mitotic cyclins. The D-box of cyclin A is 10-20 residues longer than that of cyclin B1 (Geley et al., 2001) and it has been proposed that the extended D-box may alter the substrate specificity of cyclin A thus influencing the timing of its destruction (reviewed in Fung and Poon, 2005). A study by den Elzen and Pines, (2001) has further demonstrated that degradation of human cyclin A occurs independently of the D-box. It would therefore appear that while the D-box is sufficient to degrade cyclin B1, additional mechanism(s) are required for the timely destruction of cyclin A.

#### *Subcellular localisation of cyclin A/Cdk2 and cyclin B1/Cdk1*

During interphase, cyclin B1/Cdk1 is predominantly localised in the cytoplasm by the cytoplasmic retention signal (CRS), which is located in the N-terminus of the cyclin B1 protein (Pines and Hunter, 1994). In addition, the cytoplasmic localisation of cyclin B1 during interphase also results from its export out of the nucleus, which involves a leucine-rich nuclear export signal (NES) which acts together with the export receptor CRM1/exportin 1 (Toyoshima et al., 1998; Hagting et al., 1998; Yang et al., 1998). Based on experiments that have shown that treatment with leptomycin B, an inhibitor of CRM1-mediated export, leads to a significant increase in the nuclear accumulation of cyclin B1, it has been proposed that cyclin B1 shuttles continuously between the cytoplasm and nucleus and that during interphase, the rate of export out of the nucleus exceeds the rate of import into the nucleus (Toyoshima et al., 1998; Hagting et al., 1998; Yang et al., 1998). Furthermore, at the onset of mitosis cyclin B1 is phosphorylated at four serine

residues within the CRS which reduces the affinity of cyclin B1 for the CRM1 export receptor, thereby reducing nuclear export and promoting nuclear accumulation (Li et al., 1997; Yang et al., 1998). Interestingly, Bassermann et al. (2005) have identified an additional mechanism that regulates the nuclear accumulation of cyclin B1. In their study they identified a novel E3 ubiquitin ligase, $SCF^{NIPA}$ which was shown to bind and target nuclear cyclin B1 for ubiquitination during interphase. In late G2, however, phosphorylation of $SCF^{NIPA}$ by an as yet unidentified kinase(s) results in its disassociation from cyclin B1, resulting in the nuclear accumulation of cyclin B1 and entry into mitosis. The importance of $SCF^{NIPA}$ was further demonstrated in the same study where cells treated with siRNA against $SCF^{NIPA}$ exhibited elevated levels of nuclear cyclin B1, premature entry into mitosis and a mitotic arrest at prometaphase (Bassermann et al., 2005). Taken together, the above studies suggest that the regulation of the subcellular localisation of cyclin B1 is complex and that it is probably regulated at multiple levels.

As is the case for cyclin B1, cyclin A also shuttles continuously between the nucleus and the cytoplasm. However the rate of cyclin A import into the nucleus exceeds the rate of its export (Jackman et al., 2002) and therefore it appears to be predominantly nuclear throughout the cell cycle. Importantly, while the mechanism by which cyclin A is exported from the nucleus is poorly understood, nuclear import of cyclin A is dependent on its association with CDK2.

***Phosphorylation and dephosphorylation of cyclin A/Cdk2 and cyclin B1/Cdk1***

Activation of the cyclin B1/Cdk1 complex has been proposed to happen by a series of phosphorylation and dephosphorylation events. As previously mentioned, cyclin B1 is synthesised during interphase where it associates with its catalytic subunit Cdk1. The cyclin B1/Cdk1 complex is kept inactive by phosphorylation of Cdk1 at Thr14 and Tyr15 by the inhibitory kinases, MYT1 and WEE1 (reviewed in Dunphy, 1994 and Morgan, 1997). While MYT1 can phosphorylate both Thr14 and Tyr15 (Mueller et al., 1995; Booher et al., 1997; Liu et al., 1997) WEE1 phosphorylates only Tyr15 (Heald et al., 1993; McGowan and Russell, 1993; Parker and Piwinica-Worms, 1992). At the G2/M transition, dephosphorylation at both Thr14 and Tyr15 by the Cdc25 phosphatase promotes activation of the cyclin B1/Cdk1 complex and entry into mitosis (reviewed in Dunphy, 1994; Lew and Kornbluth, 1996 and Takizawa and Morgan, 2000). Full activation of the complex is achieved by phosphorylation at Thr161 by the Cdk-activating kinase (CAK) (Solomon et al., 1992, 1993), while de-phosphorylation at this residue by KAP (CAK-associated phosphatase) returns the kinase to its inactive state in G1 (Hannon et al., 1994).

Activation of the cyclin A/Cdk2 complex also requires a series of phosphorylation and dephosphorylation events. Similar to cyclin B1/CDK1, Cyclin A/CDK2 is kept inactive by phosphorylation of CDK2 on Thr14 and Tyr15 prior to entry into mitosis (Gu et al., 1992). Full activation of the complex is achieved by phosphorylation of Thr160 by the CAK together with dephosphorylation of CDK2 at Thr14 and Tyr15 by the CDC25 phosphatase (Gu et al., 1992). Dephosphorylation of Thr160 by KAP inhibits the Cdk2 activity upon subsequent association with cyclin A (Poon and Hunter, 1995; reviewed in Lew and Kornbluth, 1996).

## 1.5 Signal transduction pathways

Signal transduction cascades mediate the sensing and subsequent movement of signals from the outside of the cell to the inside. These signalling cascades require an ordered sequence of biochemical reactions to detect, amplify and finally translate the signal into a specific cellular response, such as changes in gene expression and enzyme activity. One of the most critical post-translational modifications controlling the regulation of cellular events in response to signalling is reversible protein phosphorylation at specific serine, threonine or tyrosine residues. The process of reversible phosphorylation is controlled by kinases and phosphatases which represent a significant portion of the human genome, highlighting the importance of these enzymes in the regulation of key cellular processes (reviewed in Manning et al., 2002). The mitogen-activated protein (MAP) kinases represent one of the most important and well characterised families of kinases, which catalyse the phosphorylation of a wide range of substrates in response to diverse stimuli (reviewed in Robinson and Cobb, 1997).

### 1.5.1 Mitogen-activated protein (MAP) kinase family

MAP kinases are activated via a highly conserved phospho-relay system composed of three sequentially activated protein kinases (Fig. 1.4a). The first kinase in this cascade is the MAP kinase kinase kinase (MAPKKK) which, in response to a specific stimulus, phosphorylates and activates the dual-specificity MAP kinase kinase (MAPKK) at specific serine/threonine residues. MAPKK in turn phosphorylates MAP kinase at threonine and tyrosine residues within the Thr-Xaa-Tyr motif, which is essential for complete activation of the MAP kinase. Once activated, the proline-directed serine/threonine MAP kinase phosphorylates a range of target proteins, many of which are transcription factors, cytoplasmic substrates and other kinases (reviewed in Robinson and Cobb, 1997 and Garrington and Johnson, 1999).

The MAP kinase family is divided into four distinct subgroups: the extracellular signal-regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), ERK5/big MAP kinase 1 (BMK1) and the p38 group of protein kinases (Fig. 1.4b). ERK family members are mainly activated by mitogenic stimuli, while the JNK and p38 MAP kinases are activated mainly in response to stress stimuli or inflammatory cytokines (reviewed in Roux and Blenis, 2004 and Kyriakis and Avruch, 2001). The recently identified subgroup, ERK5, is activated by a combination of mitogenic stimuli and osmotic stress (reviewed in Wang and Tournier, 2006). The four MAP kinase subgroups differ from one another structurally in two respects (Fig 1.5). Firstly, the variable amino acid between the Thr and Tyr of the phosphorylation motif is a glutamate for ERK, proline for JNK and glycine for the p38 MAP kinases (reviewed in Ono and Han, 2000). Secondly, all three phosphorylation motifs form part of the linker loop 12 region and it is the length of the loop that varies among the different members of the MAP kinase family (Jiang et al., 1997b). In keeping with the focus of this thesis, the next section focuses on the unique characteristics and biological functions of the p38 MAP kinase family.

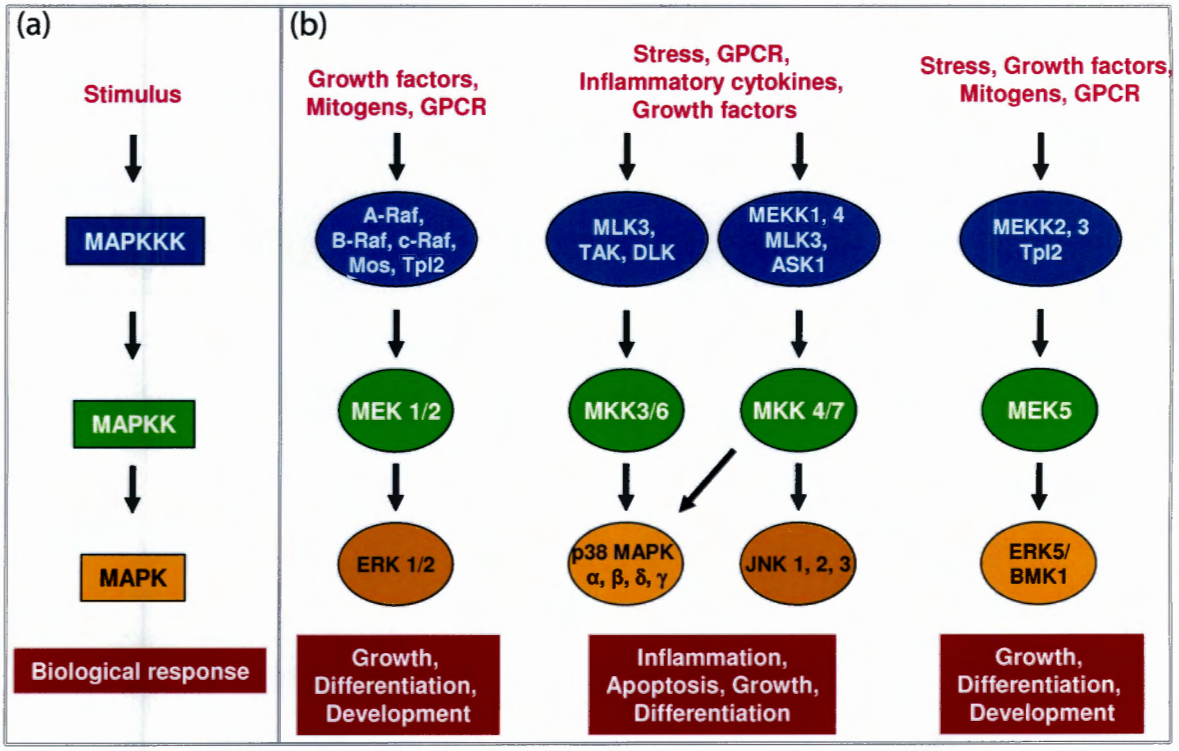


**Figure 1.4.** The MAP kinase signalling pathways. (a) The MAP kinase phospho-relay system. MAP kinase pathways are composed of three kinases: a MAP kinase (MAPK), MAPK kinase (MKK), and a MAPK kinase kinase (MKKK). (b) The MAP kinase family consists of four major subgroups: ERK1/2, p38, JNK, and ERK5/BMK1, which are activated through phosphorylation by their specific MAPKKs. MAPKKs are in turn activated through phosphorylation by their specific MAPKKKs in response to stimulation by growth factors, cytokines and cellular stress. Activated MAPKs phosphorylate various substrates including transcription factors, kinases and cytoskeletal proteins, which is critical for mediating the appropriate biological response.

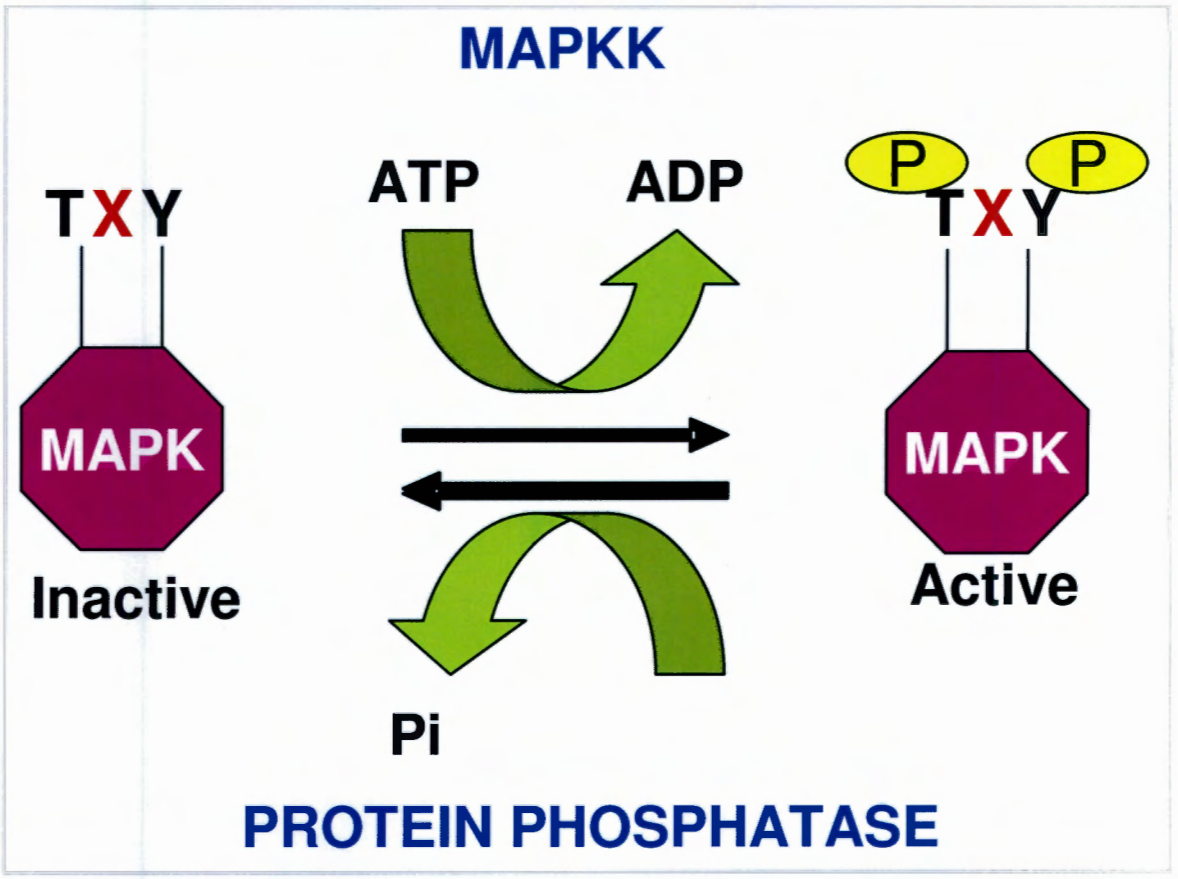


**Figure 1.5.** Dual phosphorylation within the phosphorylation motif activates the MAP kinase. Phosphorylation at both the threonine and tyrosine residues within the phosphorylation motif (TXY) by the dual-specificity MAPKK activates the MAP kinase. Specific protein phosphatases inactivate the MAPK by dephosphorylating either one or both of the phosphorylated residues. The variable amino acid, X, may be either glutamate for ERK, proline for JNK and glycine for the p38 MAP kinase.

### 1.5.2 p38 MAP kinase signalling pathway

The p38 MAP kinase (referred to as p38), also known as reactivating kinase (RK), cytokine synthesis anti-inflammatory drug (CSAID) binding protein (CSBP) or stress-activated protein kinase 2a (SAPK2a), is the mammalian homologue of the yeast HOG kinase (Rouse et al., 1994; Lee et al., 1994). p38 was first identified as a 38 kDa protein (Han et al., 1993, 1994) and to date four primary isoforms of p38 have been characterised: p38α, p38β, p38γ and p38δ. The p38α and p38β isoforms are ubiquitously expressed (Jiang et al., 1996), whereas p38γ is predominantly expressed in skeletal muscle (Lechner, 1996; Li et al., 1996) and p38δ is detected in the lungs, kidneys, testis, pancreas and small intestine (Kumar et al., 1997). More than 60% sequence identity exists between each isoform, with only 40-45% sequence identity observed among other MAP kinase members (Jiang et al., 1997a). Tissue distribution and/or expression patterns reflect differences in the regulation and functions of each p38 isoform.

Once activated p38 has been shown to phosphorylate a range of protein kinases and transcription factors involved in both stress and non-stress cellular processes (reviewed in Zarubin and Han, 2005 and Nebreda and Porras, 2000). The cellular stresses that activate the p38 signalling pathway include osmotic shock, ultraviolet (UV) light, heat shock, protein synthesis inhibitors, inflammatory cytokines and liposaccharides (reviewed in Zarubin and Han, 2005). In response to cellular stress the p38 pathway impacts on a number of cellular processes including proliferation, survival, cell differentiation, the inflammatory response and apoptosis. p38 also plays a role in non-stress cellular processes, for example during embryonic development, differentiation, proliferation and survival of several cell types (reviewed in Bradham and McClay, 2006 and Nebreda and Porras, 2000).

#### 1.5.2.1 Regulation of the p38 MAP kinase

While the total level of p38 protein remains unchanged, in response to a specific stimulus, phosphorylation at Thr180 and Tyr182 by the dual-specificity kinases MAPKK3, MAPKK4, MAPKK6 and MAPKK7 (Doza et al., 1995; Raingeaud et al., 1995; Dérijard et al., 1995; Han et al., 1996) leads to increased levels of phosphorylated p38. This phosphorylation is often transient and results in rapid activation of p38. Despite the high degree of homology between the various p38 isoforms and the conserved dual phosphorylation site, they are selectively activated by different MAPKKs (Fig. 1.4b). For example, while MAPKK6 is able to activate all the p38 isoforms, MAPKK3 is unable to activate p38β (Keesler et al., 1998) and MAPKK7 has also been shown to activate p38δ (Hu et al., 1999). Furthermore, MAPKK4, an upstream activator of JNK, has also been found to activate p38α and p38δ in a cell-type specific manner (Jiang et al., 1997a).

In response to several physiological stimuli, such as tumour necrosis factor (TNF) and CpG oligonucleotide treatment, p38α can also be activated by phosphorylation at Thr180 and Tyr182 by transforming growth factor- β-activated protein kinase 1 (TAB1) (Ge et al., 2002, 2003). When associated with TAB1, p38 therefore appears to be able to autophosphorylate itself. In contrast, a study using MAPKK3/6 knockout MEF cells showed that phosphorylation of p38 in response to TNF and UV radiation was dependent on MAPKKs (Brancho et al., 2003).

In addition, a third mechanism of activation for p38 has been identified in T cells. In response to T cell receptor stimulation, both ZAP-70 and Src family tyrosine kinases phosphorylate p38α and p38β on Tyr323, which induces autophosphorylation at the dual phosphorylation sites (Salvador et al., 2005). These studies suggest that alternative activation pathways of p38 may exist in response to different physiological and pathological conditions. There are also additional mechanisms ensuring the efficacy and specificity of the signal transduced by the p38 kinase into the appropriate cellular response. These include scaffold proteins, docking interactions, subcellular distribution, protein phosphatases and cross-talk among MAP kinases and other signalling pathways.

***Scaffold proteins and Docking interactions***

Recent studies have identified scaffold proteins as playing an important role in regulating the activation of specific MAP kinase signalling pathways. Scaffold proteins ensure that specific signalling pathways are triggered by recruiting active enzymes into appropriate signalling cascades by localising enzymes to specific subcellular compartments or substrates (reviewed in Whitmarsh and Davis, 1998). The p38 and JNK signalling pathways represent an example of where scaffold proteins play an important role in determining which pathway will be activated in response to cellular stress. The JNK Interacting Proteins (JIP) constitutes a family of scaffold proteins that interact with both the JNK and p38 pathways. The JIP proteins promote p38 signalling by binding p38 directly or components of the p38 signalling pathway. For example, JIP2 is able to bind the p38α and p38γ isoforms, MKK3 and the Rac-GTPase (Buchsbaum et al., 2002; Schoorlemmer and Goldfarb, 2001). The molecular mechanism of how JIP family members are able to selectively regulate both JNK and p38 are not known but one possibility is that JNK and the p38 isoforms may compete for binding to JIP proteins, thereby inhibiting the activation of each other (reviewed in Whitmarsh, 2006).

Together with scaffold proteins, the efficacy and specificity of the protein-protein interactions between p38 and its interacting molecules and phosphatases are mediated by docking interactions. Docking interactions are distinct from the kinase catalytic centre and the dual phosphorylation motif (reviewed in Tanoue and Nishida, 2002, 2003). Briefly, two reciprocal docking sites on the surface of p38, called the common docking (CD) domain and the ED site, mediate binding to the docking (D) domain present in p38 activators and downstream substrates (Fig. 1.6). The CD domain is conserved among all MAP kinases at the C-termini and contains both acidic and hydrophobic amino acids (Tanoue et al., 2000). The acidic residues (Asp313, Asp315 and Asp316) in the human p38α interact with the positively charged residues in the D domain of p38 substrates and phosphatases. In addition to the CD domain, the ED site (Glu160 and Asp161), is also important in substrate recognition (Tanoue et al., 2001). Together, the CD domain and the ED site form the docking groove of p38 and each amino acid residue in the docking groove contributes differently to specific docking interactions (Tanoue et al., 2001).

It is important to note that a subset of MAP kinase substrates also have a conserved motif (FXFP) located C-terminally to the MAP kinase phosphorylation site, which is required for

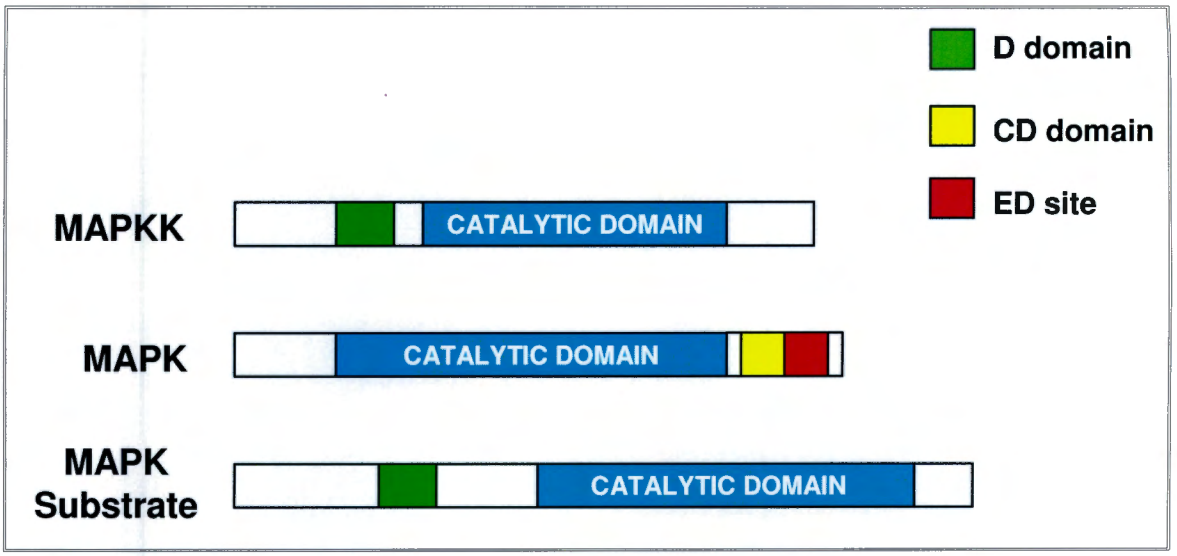


**Figure 1.6.** Schematic representation of the docking domains of MAPKK, MAPK and the MAPK substrate. The positively charged residues in the docking (D) domain interact with the common docking (CD) domain and ED site in the C-terminal region of the MAP kinase. The CD and ED site form a docking groove where each amino acid in the groove contributes differently to each docking interaction.

efficient phosphorylation by MAP kinases including p38 (Galanis et al., 2001). Residues within the activation loop of MAP kinases have also been found to be important in substrate specificity. For example, MKK3 does not possess a D domain and yet is able to activate p38α, p38γ and p38δ but not p38β (Enslen et al., 2000). Interestingly, when the activation loop of p38β was mutated into a p38α, p38γ and p38δ-like activation loop it was activated by MKK3. These results suggest that residues within the activation loop are also able to promote activation of the p38 isoforms (Enslen et al., 2000).

***Subcellular localisation***

Given the fact that p38 phosphorylates various substrates in distinct cellular compartments, controlling its subcellular localisation is important. Despite the evidence for the nucleocytoplasmic shuttling of p38, no NLS or nuclear export signals (NES) have been identified for p38 or other members of the MAP kinase family. Furthermore, no direct interactions between MAP kinases and importins or exportins have been detected. Shuttling of p38 across the nuclear membrane is most likely a result of specific interactions with NES- and NLS containing proteins, such as p38 activators, substrates or scaffold proteins (reviewed in Cyert, 2001). Finally, docking interactions can also regulate the subcellular localisation of MAP kinase and MAP kinase substrates. For example, p38 can form a stable complex with its substrate, MAPKAP-2, which contains a NLS sequence that permits the translocation of the MAPKAP-p38 complex from the nucleus (Ben-Levy et al., 1998).

***Protein phosphatases***

Inactivation of p38 occurs rapidly by dephosphorylation at either phospho-Thr180 or phospho-Tyr182 by a family of protein phosphatases, called MAP kinase phosphatases (MKPs). MKPs are divided into three classes based on their preference for dephosphorylating tyrosine, serine/threonine or both tyrosine and threonine termed dual specificity phosphatases (DSPs) (reviewed in Keyse, 2000). Recently, there has been significant progress in understanding the regulation and role of DSPs (reviewed in Camps et al., 2000 and Farooq and Zhou, 2004). In response to a specific stimulus, a subset of rapidly transcribed and translated DSPs translocates to a specific subcellular compartment within either the nucleus or cytoplasm. The DSPs become catalytically active upon binding via their amino-terminal domain to the target MAP kinase. If the bound MAP kinase is active, it will become rapidly dephosphorylated by the DSPs. However, if the MAP kinase is inactive, binding by the DSPs will prevent activation by any subsequent stimulus.

Certain DSP family members display specificity for dephosphorylating different p38 isoforms. For example, whereas MAP kinase phosphatase-1 (MKP-1), MKP-4 and MKP-5 can efficiently dephosphorylate p38α and p38β (Chu et al., 1996; Muda et al., 1997; Tanoue et al., 1999), p38γ and p38δ are resistant to all MKP family members. Similarly, other types of phosphatases, such as the serine/threonine protein phosphatase type 2C (PP2C), have been shown to downregulate upstream activators of the p38 isoforms such as MKK6 and MKK4 both in vitro and in vivo (Takekawa et al., 1998). It is speculated that this may be a mechanism to differentially regulate the p38 isoforms in distinct tissue, cell and subcellular compartments.

### 1.5.2.3 Synthetic inhibitors of the p38 MAP kinase

The p38 signalling pathway has been identified to play an important role in a number of inflammatory diseases such as rheumatoid arthritis, Alzheimer's disease and inflammatory bowel disease (reviewed in Westra and Limburg, 2006; Johnson and Bailey, 2003 and Peifer et al., 2006). During the inflammatory response, the p38 pathway is activated, leading to the production of proinflammatory cytokines and other proteins involved in mediating the inflammatory response (Lee et al., 1994; reviewed in Kyriakis and Avruch, 2001). Inhibition of the p38 pathway was therefore an obvious target for therapy of chronic inflammatory disease. The early inhibitors of p38 were pyridinyl imidazole compounds of which the most widely used is SB203580 and SB202190 (Badger et al., 1996; Lee at al., 1994). These inhibitors function by binding the active site of active and inactive forms of p38 thereby inhibiting the enzymatic activity of the kinase (Young et al., 1997; Frantz et al., 1998). Both SB203580 and SB202190 are selective for p38α and p38β, and X-ray crystallography showed that Thr106 present in their ATP-binding domain is important for this selectivity (Tong et al., 1997; Kumar et al., 1997; Young et al., 1997; Lisnock et al., 1998). In addition to their therapeutic benefits, the SB203580 and SB202190 inhibitors have also proven to be powerful experimental tools to distinguish between the roles of the p38 kinase and JNK in the stress response.

### 1.5.2.4 DNA damage and the role of the p38 MAP kinase

UV irradiation, a known mutagen and carcinogen, is a major causative agent in skin cancer because of its ability to induce DNA damage (reviewed in Cadet et al., 2005). The main mechanism of UV-induced DNA damage is the formation of DNA photoproducts, with the most common being pyrimidine dimers (CPD) and 6-4 photoproducts, which form at sites of adjacent pyrimidine bases. The intrastrand linkage between adjacent pyrimidines (usually thymidines), creates a distortion in the double helix thereby affecting replication and transcription.

The DNA damage induced by UV radiation triggers a distinct cellular response which involves activation of the DNA damage checkpoints and stress signalling pathways. A key player mediating the cells response to DNA damage is the tumour suppressor protein, p53. Once activated p53 can arrest the cells at G1 and G2 to allow for repair of the damaged DNA, but if the damage is irreparable, p53 may either induce the cells to senesce or undergo apoptosis. p53 triggers a G1 arrest by, in part, inducing the expression of the CDKi, p21 (el Deiry et al., 1993) which binds and inhibits the activity of the G1 cyclin/Cdk complexes (Xiong et al., 1993; Harper et al., 1993, 1995; reviewed in Sherr and Roberts, 1999). In response to DNA damage, p21 can also block cell cycle progression by inducing a G1 and G2 arrest independent of p53 (reviewed in Giono and Manfredi, 2006). The key role of p21 in inducing a G1 arrest in response to DNA damage was illustrated in studies which have shown that MEFs deficient for p21 exhibit an impaired G1 arrest, and deletion of p21 in a colon cancer cell line ablated the G1 arrest in response to different DNA damaging agents (Brugarolas et al., 1995; Deng et al., 1995; Waldman et al., 1995).

In response to DNA damage, p53 also plays a role at the G2/M checkpoint where it is required for the maintenance rather than the initiation of the G2 arrest. For example, in response to

ionizing radiation, wild type p53 cells arrest in G2 whereas mutant p53 cells can initially arrest but eventually escape and enter mitosis (Bunz et al., 1998). During G2 p53 halts cell cycle progression by directly repressing the expression of the *Cdc25c* phosphatase (St. Clair et al., 2004) and by upregulating the expression of *14-3-3σ* (Hermeking et al., 1997) which functions to sequester Cdc25c in the cytoplasm, preventing it from activating the cyclin B1/CDK1 (Dalal et al., 1999). A number of different mechanisms have been identified for the involvement of p21 in arresting cells in G2 in response to DNA damage. During late G2, in response to DNA damage p21 binds inactive cyclin A/Cdk2 and to a lesser extent cyclin B1/Cdk1 complexes and this interaction is thought to delay entry into mitosis (Dulić et al., 1998). Furthermore, when cells lacking either p53 or p21 are gamma-irradiated they enter mitosis but are unable to complete cytokinesis, and subsequently either exhibit features of chromosomal instability or undergo apoptosis (Bunz et al., 1998). Ando et al. (2001) has also shown that the interaction between p21 and the proliferating cell nuclear antigen (PCNA) is required to maintain this G2 arrest. They show that in the absence of DNA damage, PCNA associates with Cdc25C which results in the activation of cyclin B1/CDK1 and hence in promoting G2/M transition. In response to DNA damage, however, p21 was found to induce a G2 arrest by complexing with PCNA and thus blocking the association of PCNA with Cdc25C. Interestingly, the p21-PCNA complex further prevents G2/M transition by sequestering cyclin B1/CDK1 and holding it inactive. Consistent with the above study, Charrier-Savournin et al. (2004) showed using normal human fibroblast cells that in response to non-repairable DNA damage, p21 retains inactive cyclin B1/CDK1 in the nucleus, thus preventing its activation and recruitment to the centrosomes. Moreover, p21 can also inhibit activation of the cyclin B1/Cdk1 complexes by preventing the CAK from activating the Cdk1 kinase (Smits et al., 2000). Taken together, p21 appears to mediate a G2 cell cycle arrest through multiple mechanisms which ultimately involve inhibiting the activity of the cyclin/Cdks and/or PCNA.

UV-induced p38 activation plays an important role in the p53-mediated cell cycle arrest described above. A number of studies have demonstrated that following UV radiation, p53 is multiply phosphorylated at serine residues by p38. In vitro and in vivo studies have shown that in response to UVB, mouse p53 is phosphorylated at serine 15 by ERK and p38 (She et al., 2000). This phosphorylation was shown by Shieh et al. (1997) to prevent binding of MDM2 to p53 and thus to increase the stability of the protein. Furthermore, UVB irradiation of normal human keratinocytes led to phosphorylation of p53 at serine 15 by p38, which was found to protect these cells from apoptosis (Chouinard et al., 2002). Interestingly, in another study, p38 was shown to phosphorylate human p53 at serine residues 33 and 46 in response to UVC radiation (Bulavin et al., 1999). Phosphorylation at these two sites was also shown to induce both the transcriptional activity of p53 as well as the ability of p53 to mediate apoptosis in response to UV radiation. Furthermore, phosphorylation by the p38 kinase at these two sites was also shown to be important for the subsequent phosphorylation of p53 at two other N-terminal residues, serine 15 and 37, in response to UV radiation. UVC induced phosphorylation of p53 at serine 389 by p38 has also been observed. Phosphorylation at this site by the p38 kinase is required for the regulation of the DNA-binding and transcriptional activity of p53 (Keller et al., 1999; Huang et al., 1999).

Depending on the degree of damage, activation of the p38-p53 signalling cascade in response to UV radiation may be transient to allow repair of the damaged DNA or prolonged to promote apoptosis. The mechanism(s) controlling the duration of activation of the p38-p53 signalling cascade therefore needs to be tightly regulated. Recently, a novel p38 regulated protein, called $p18^{Hamlet}$ was identified as an important co-activator of p53 (Cuadrado et al., 2007). In response to UV, phosphorylation by the p38 MAP kinase leads to increased stability of $p18^{Hamlet}$, which enhances its association with p53 to stimulate the transcription of several of its proapoptotic target genes, such as the p53 upregulated modulator of apoptosis (PUMA) and NOXA (Latin meaning "harm" or "damage"). A study by Takekawa et al. (2000) on the other hand, has described a mechanism which leads to the downregulation of the p38-p53 signalling pathway during the recovery period following UV radiation. They show that the Wip1 phosphatase dephosphorylates and consequently inactivates p38, which selectively inhibits its ability to phosphorylate p53 thereby suppressing both p53-mediated transcription and apoptosis in response to UV radiation.

The p38 signalling pathway has also been shown to play a role at the G2 checkpoint in response to UV irradiation which is independent of p53 (Bulavin et al., 2001). The ability of p38 to phosphorylate Cdc25B phosphatase was identified as one molecular mechanism for the UV-induced G2 arrest. In vitro, p38 was shown to bind and phosphorylate Cdc25B at serine 309 (Bulavin et al., 2001) and serine 323 (Lindqvist et al., 2004), which results in it binding to the 14-3-3 proteins and thus preventing its translocation into the nucleus, where it is required to activate the cyclin B1/CDK1 complex (Bulavin et al., 2001). Interestingly, a recent study by Manke et al. (2005) identified a different mechanism for the G2/M arrest in response to UVC irradiation. This mechanism involves the downstream substrate of p38, MAPKAP K2. In response to UV-induced damage MAPKAP K2 induces cell cycle arrest by phosphorylating Cdc25B and C which induces binding of 14-3-3. They show using RNAi that endogenous MAPKAP K2 is responsible for inducing not only G2 but also the G1 and S checkpoints in response to UV irradiation (Manke et al., 2005). The investigators of this study argue that as p38 requires MAPKAP K2 to translocate from the nucleus (Ben-Levy et al., 1998) and since the experiments by Bulavin et al. (2001) involved immunoprecipitating p38 from the nucleus, they may have overlooked the presence of MAPKAP K2 in their immunoprecipitates.

## 1.6 General and specific aims of study

During development, Tbx2 has been implicated in several processes such as coordinating cell fate, patterning and morphogenesis of a wide range of tissues and organs. Furthermore, Tbx2 is also emerging as a key regulator of the cell cycle and dosage sensitivity of this gene has been implicated in several cancers. However very little is also known about the biochemical pathways regulating the expression and activity of Tbx2, and while its role as a transcriptional repressor is well defined there is limited information regarding its target genes. The identification of kinases and signalling pathways regulating the activity of Tbx2 is imperative in order to elucidate the precise role of Tbx2 in the cell cycle, which may shed light on its oncogenic role.

Preliminary work in our laboratory, in collaboration with Dr Colin Goding (Marie Curie Research Institute, UK), has previously shown that Tbx2 is phosphorylated by the p38 MAP kinase and the mitotic cyclin/Cdk complexes.

The **specific aims** of this study were:

1. to determine whether Tbx2 is phosphorylated by the p38 MAP kinase, cyclin A/Cdk2 and cyclin B1/Cdk1 both in vitro and in vivo
2. to map the specific sites within Tbx2 targeted by p38 MAP kinase, cyclin A/Cdk2 and cyclin B1/Cdk1
3. to determine the functional consequence of Tbx2 phosphorylation by the identified kinases by testing the affect of phosphorylation on:
   (a) the protein stability of Tbx2
   (b) the subcellular localisation of Tbx2
   (c) the ability of Tbx2 to repress transcription of the *p21* gene

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Plasmids and DNA constructs

All constructs used in this study were prepared according to standard techniques (Sambrook et al., 1989, pp 1.25-1.51). The wild-type (WT) p21-luciferase (LUC) construct contains a 2.2 kb *Sac* I- *Hind* III fragment of the human *p21* promoter inserted upstream of a LUC reporter gene (el Deiry et al., 1993). As has previously been described the pCMV19.Tbx2 and pGEX. Tbx2 were constructed by cloning a full-length *Bam* HI fragment of the mouse *Tbx2* cDNA into the *Bam* HI site of the pCMV19a and pGEX.2TK vectors, respectively (Carreira et al., 1998). The GST-cyclin A and B1 fusion constructs and T7-plink Tbx2 full-length and deletion constructs were kindly provided by Dr C. Goding (Marie Curie Research Institute, UK). The pGL3-Basic-p14$^{ARF}$ (642) luciferase construct was kindly provided by Dr D. Holzschu (Ohio University, Athens, Ohio).

WT Tbx2 and Tbx2 point mutants were cloned as *Bam* HI fragments into the polylinker of the pGEX.2TK expression vector 3' to the GST. Briefly, 10 µg pCMV19.Tbx2 was digested with 0.5 units/µl *Bam* HI (Roche, Switzerland) at 37°C for 3 hours (hr). The DNA was then precipitated overnight in 3 M NaOAc (1/10 volume) and 100% ice-cold ethanol (2.5X) at -20°C. The DNA was collected by centrifugation at 14 000 rpm for 15 min at room temperature (RT) and the pellet air-dried and resuspended in 30 µl $dH_20$. The DNA was electrophoresed on a 0.8% agarose gel and the *Bam* HI fragment of the full-length Tbx2 was directly purified from the agarose gel using the Wizard SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manufacturer's instructions.

To generate the N-terminal (1-371 amino acids) Tbx2 protein, the *Bgl* II-*Eco* RI *Tbx2* cDNA fragment (1140-2281 bp) was released from the pGEX.Tbx2 plasmid with appropriate enzymes. To ensure efficient religation of the remaining plasmid, the blunt-ended *Bgl* II and *Eco* RI digested plasmid was incubated with 0.5 units/µl MuLV reverse transcriptase (Applied Biosystems, CA, USA) for 30 min at 37°C. The plasmid was precipitated overnight by adding 200 µl $dH_20$, 3 M NaOAc (1/10 volume) and 100% ice-cold ethanol (2.5X) at -20°C. To generate the C-terminal (371-711 amino-acids) Tbx2 protein, the *Tbx2* cDNA fragment (1140-2281 bp) was subcloned as a *Bgl* II-*Eco* RI fragment into the pGEX.2TK expression vector.

## 2.2 Site-directed mutagenesis of the mouse *Tbx2* cDNA

Point mutations were introduced into the pCMV19.Tbx2 expression construct containing the full-length WT *Tbx2* cDNA by site-directed mutagenesis using the Stratagene QuikChange system (California, USA) and appropriate primer pairs containing the desired mutations (see Table 2.1). Standard polymerase chain reaction (PCR) mix contained: 1X Reaction buffer, 25 ng ds DNA template, 125 ng Forward primer, 125 ng Reverse primer, 1 µl dNTP mix, 4 µl Dimethyl Sulphoxide (DMSO) (Sigma, St. Louis, MO, USA), 1 µl *PfuTurbo* DNA polymerase (2.5 U/µl) in a total volume of 50 µl. The parameters for PCR amplification were 30 seconds (sec)

**Table 2.1.** Sequence of primers used to generate mutant *Tbx2* cDNA constructs by site-directed mutagenesis[a]

| Mutant generated | Mutation | ds DNA template | Forward primer | RE site introduced |
|---|---|---|---|---|
| S192A | S192A | WT Tbx2 | 5'-CATCCACCCGGAC**gct**CCGGCCACCGGTGAACAGTGGATG-3' | *Age* I |
| S192E | S192E | WT Tbx2 | 5'-CATCCACCCGGAC**gag**CCGGCCACCGGTGAACAGTGGATG-3' | *Age* I |
| S336A | S336A | WT Tbx2 | 5'-GAACCACCGCCC**gcc**CCCAGCGCTGCGCCCAGCCCC-3' | *Eco47* III |
| S336E | S336E | WT Tbx2 | 5'-GAACCACCGCCC**gag**CCCAGCGCTGCGCCCAGCCCC-3' | *Eco47* III |
| S192A:S336A | S336A | S192A | 5'-GAACCACCGCCC**gcc**CCCAGCGCTGCGCCCAGCCCC-3' | *Eco47* III |
| S192E:S336E | S336E | S192E | 5'-GAACCACCGCCC**gag**CCCAGCGCTGCGCCCAGCCCC-3' | *Eco47* III |
| S342A | S342A | WT Tbx2 | 5'-CCCTCCCCCAGCGCTGCGCCC**gcc**CCTCTGCGCCTAC-3' | *Eco47* III |
| S342E | S342E | WT Tbx2 | 5'-CCCTCCCCCAGCGCTGCGCCC**gag**CCTCTGCGCCTAC-3' | *Eco47* III |
| S336A:S342A | S336A:S342A | WT Tbx2 | 5'-GAACCACCGCCC**gcc**CCCAGCGCTGCGCCC**gcc**CCTCTGCGCC-3' | *Eco47* III |
| S336E:S342E | S336E:S342E | WT Tbx2 | 5'-GAACCACCGCCC**gag**CCCAGCGCTGCGCCC**gag**CCTCTGCGCC-3' | *Eco47* III |
| S623A | S623A | WT Tbx2 | 5'-GGGCAGTGCCAGGCCTCGTCTGCGCTTC**gct**CCTTACCAG-3' | *Stu* I |
| S623E | S623E | WT Tbx2 | 5'-GGGCAGTGCCAGGCCTCGTCTGCGCTTC**gag**CCTTACCAG-3' | *Stu* I |
| S675A | S675A | WT Tbx2 | 5'-CGCGGGGCCCTA**gcg**CCCTCCGGATCAGCCAAAG-3' | *BspE* I |
| S675E | S675E | WT Tbx2 | 5'-CGCGGGGCCCTA**gag**CCCTCCGGATCAGCCAAAG-3' | *BspE* I |
| S336A:S623A:S675A | S336A | S623A:S675A | 5'-GAACCACCGCCC**gcc**CCCAGCGCTGCGCCCAGCCCC-3' | *Eco47* III |
| S336E:S623E:S675E | S336E | S623E:S675E | 5'-GAACCACCGCCC**gag**CCCAGCGCTGCGCCCAGCCCC-3' | *Eco47* III |

[a]*Primer* sequences corresponding to the template *Tbx2* cDNA are represented in uppercase. Mutations converting serine to either alanine (GCC, GCT or GCG) or glutamic acid (GAG) are depicted in bold lower case. Restriction enzyme sites introduced via the mutation are underlined with the point mutation in bold. Mutants generated in the present study are highlighted in blue font.

at 95°C for 1 cycle, 30 sec at 95°C, 1 minute (min) at 55°C and 12 min at 68°C for 16 cycles. PCR was performed on a Hybaid PCR Express Thermal cycler (Middlesex, UK). The PCR products were assessed by agarose gel electrophoresis to confirm successful amplification. The synthesised DNA containing the mutation was selected for by digesting the methylated, non-mutated parental DNA template with 1 µl *Dpn I* (10U/µl) endonuclease for 1 hr at 37°C. XL1-Blue supercompetent cells (Stratagene, USA) were transformed with 1 µl of the *Dpn I* digested DNA as follows: incubation on ice for 30 min, heat pulsing at 42°C for 45 sec and ice treatment for 2 min. To each transformation reaction 0.5 ml of NZY+ broth (see Appendix), preheated to 42°C, was added and then incubated for 1 hr at 37°C with shaking at 225-250 rpm. Following incubation, the entire volume of each transformation reaction was plated onto Luria broth Ampicillin (50 µg/ml) agar plates. Plates were incubated at 37°C for at least 16 hrs. To confirm successful transformation of the mutated plasmid DNA, the presence of the mutation was verified by restriction enzyme analysis (see Table 2.1) and sequencing.

## 2.3 Cell culture

### 2.3.1 Maintenance of cells in culture

COS-7 monkey kidney cells, NIH 3T3 mouse fibroblasts, WI-38 human lung fibroblasts, HT1080 human fibrosarcoma cells, B1Tbx2 mouse 3T3 fibroblast cells stably expressing SV-5 tagged Tbx2 and the CT-1 transformed human lung fibroblast cell line stably expressing TBX2 (CT-Tbx2, Davis et al., 2007) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200 units/ml penicillin and 100 µg/ml streptomycin. Mouse B16 melanoma cells were grown in RPMI 1640 supplemented with 10% FBS, 200 units/ml penicillin and 100 µg/ml streptomycin and the MCF-7 human breast epithelial adenocarcinoma cell line, was cultured in RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids and 0.01 mg/ml insulin. Cells were maintained in a 37°C incubator (95.0% air/5% CO2, 65% humidity) and cell media were replaced every 2-3 days. All cultures were routinely subjected to mycoplasma tests and only mycoplasma free cells were used in experiments.

### 2.3.2 Mycoplasma test

Cells grown on a coverslip in antibiotic-free media for 2-3 days were fixed in a 1:3 mixture of glacial acetic acid and methanol for 5 sec, washed briefly with water to remove the fixing solution and then air-dried at RT for 5 min. Once dried, the DNA was stained with Hoechst 33258 (0.5 µg/ml) for 30 sec, washed briefly with water to remove excess stain and then mounted on a slide with mounting fluid (see Appendix) at pH 5.5. The cells were viewed immediately by fluorescence microscopy under the DAPI filter. Mycoplasma negative cells stained positive with Hoechst 33258 only in the nucleus, while cells infected with mycoplasma showed staining in both the nucleus and cytoplasm.

### 2.4.5 Phosphatase treatment

Prior to SDS-Polyacrylamide Gel Electrophoresis (PAGE), 30 µg MCF-7 whole cell lysates were either incubated in the presence of one unit of shrimp alkaline phosphatase (USB, Cleveland, OH, USA) at 37°C for 30 min or were not treated with phosphatase. The reaction was stopped by adding 5X SDS loading buffer (see Appendix) and boiled at 100°C for 5 min.

## 2.5 Transfection assays

Prior to performing transfections, the concentration and quality of all DNA constructs was confirmed by agarose gel electrophoresis (data not shown). COS-7 and NIH 3T3 cells were transiently transfected with FuGENE 6 (Roche, Switzerland) and/or FuGENE HD (Roche, Switzerland) using a 3:1 ratio according to the manufacturer's instructions. Cells were plated at 0.5 X $10^5$/ml in 35 mm dishes 1 day before transfection. Three microlitres of the transfection reagent diluted into 97 µl serum-free media (without antibiotic) was prepared at RT and allowed to stand for 5 min. The diluted transfection reagent was added in a dropwise manner to the DNA, mixed and incubated at RT for 15 min. Next the transfection reagent:DNA complex was added dropwise to the cells and thereafter incubated for thirty hours in a 37°C incubator. COS-7 and NIH 3T3 cells were transfected with 0.5 µg and 1 µg DNA, respectively.

## 2.6 Luciferase assays

COS-7 cells were transfected with 700 ng of the p21 or $p14^{ARF}$ luciferase reporter plasmid plus 200 ng of the Tbx2 expression plasmid or 200 ng of an empty vector plasmid (pCMV19). The vector pRL-TK, containing the thymidine kinase promoter which drives the expression of a *renilla* reporter, was used as an internal control for transfection efficiency (50 ng per transfection). Cells were cultured for 30 hr and extracts were then assayed for firefly and *renilla* luciferase activity using the dual luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells cultured in a 35 mm dish were lysed using 1X lysis buffer. Cell lysates subjected to two freeze-thaw cycles were collected by centrifugation at 13 000 rpm for 1 min at RT. The supernatant was transferred to a fresh eppendorf and 10 µl of cell lysate was assayed immediately for reporter gene activity. Luciferase activities were measured using the Luminoskan Ascent luminometer (Thermo Labsystems, Franklin, MA, USA). To normalise transfection efficiency, firefly luciferase values were divided by the *renilla* luciferase activity. Promoter activity was calculated as a ratio of the luciferase activity generated by the empty vector (without Tbx2) to that obtained in the presence of the appropriate expression vector. All luciferase assays were performed in duplicate and at least three independent experiments were done to confirm reproducibility. The Microsoft Excel programme was used to calculate the standard deviation and statistically significant differences between samples using the Student *t* test. *P* values of $<0.05$ were considered statistically relevant.

## 2.7 Western blot analyses

Cells were harvested by trypsinisation, washed twice with ice-cold PBS and collected by scraping with a 1 ml plunger. Whole cell extracts prepared from cells using RIPA buffer (see

Appendix) were stored on ice for 30 min and collected by centrifugation at 12 000 rpm for 20 min at 4°C. The protein concentration for each cell extract was determined using the BCA Protein Assay kit (Pierce, USA), with bovine serum albumin as the standard. Equal amounts of protein were loaded in each lane and resolved on 7.5-15% SDS-PAGE gels and then transferred electrophorectically to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, USA). Following exposure to blocking solution (see Appendix) for 1 hr at RT with shaking, the membrane was incubated with the appropriate primary antibodies overnight at 4°C. Following this, the membrane was incubated with either goat anti-mouse or goat anti-rabbit IgG (H+L)-HRP conjugated secondary antibodies (1:5000) (BioRad, Hercules, CA, USA) at RT with shaking for 1 hr. Proteins of interest were detected using the SuperSignal Chemiluminiscent system (Pierce, USA). Primary antibodies and appropriate dilutions were: mouse monoclonal anti-Tbx2 62-2 antibody (1:2000), rabbit polyclonal anti-phospho-p38 (1:1000) (Cell Signaling Technology Inc., Beverly, MA), rabbit anti-p38 (1:5000) (Sigma, Missouri, USA), anti-Pk-Tag (SV5) antibody (1:3000) (Serotec, Oxford, UK) and rabbit polyclonal anti-p21 (1:200), anti-cyclin A (1:1000), anti-cyclin B1 (1:1000) and mouse monoclonal anti-α-tubulin (1:500) all from Santa Cruz Biotechnology, CA, USA.

## 2.8 Immunofluorescence

Cells, grown on coverslips in 35mm dishes were washed with PBS and then fixed with 4% paraformaldehyde for 20 min at RT. Thereafter the cells were permeabilised in 0.2% Triton X-100/PBS at RT for 10 min. Following permeabilisation, cells were blocked for 1hr in 5% swine serum in PBS at RT. After overnight incubation at 4°C with the appropriate antibodies (diluted in PBS and 5% swine serum), cells were washed and incubated with the appropriate fluor-conjugated secondary antibodies (1:1000 dilution) for 90 min in the dark at RT. For DNA staining, cells were incubated with 1 µg/ml DAPI (4',6-diamidino-2-phenylindole) (Sigma, Germany) diluted in PBS for 10 min at RT in the dark. Cells were washed and then mounted on slides with Mowial mounting medium (Hoechst, Germany) containing Anti-Fade (Sigma, USA). Fluorescent cells were viewed using standard FITC and DAPI filters on an Axiovert fluorescent microscope (Zeiss, USA). Primary antibodies (have been described in 2.7): anti-Tbx2 antibody (1:750), anti-phospho-p38 (1:1000) and rabbit polyclonal anti-phospho-Histone H3 (1:200) (Upstate Biotechnology, Charlottesville, USA). Secondary antibodies used were: alexa 488 goat anti-mouse (Molecular Probes, Eugene, USA) and Cy3 donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., USA). Negative controls had primary antibody excluded.

## 2.9 Bacterial expression and purification of glutathione S-transferase (GST) fusion proteins

All GST-fusion proteins were prepared in the pGEX.2TK vector as previously described (see 2.1). The GST-fusion proteins were transformed into the *Escherichia coli* (*E. coli*) strain pLysS and protein expression induced for 4 hr with 0.5 M isopropyl-1-thio-D-galactopyranoside (IPTG) (Promega, USA) at 37°C with shaking. Bacterial cells were collected by centrifugation at 3 000 rpm for 30 min at RT and the resulting pellet resuspended in 1 ml PBSTi buffer (see Appendix)

was lysed by sonication. Cellular debris was removed by centrifugation at 13 000 rpm for 30 min at 4°C. The protein lysates were incubated with 140 µl/PBSTi Glutathione Sepharose 4B beads (Amersham Biosciences, USA) for 1 hr at 4°C with rolling. The bead-bound complexes were washed four times in 1 ml PBSTi buffer. Following washes, the purified proteins were resuspended in 200 µl PBSTi and stored at 4°C. Quantification was accomplished by resolving equal volumes of GST and GST-fusion protein on a 12% SDS-PAGE followed by Coomassie staining.

## 2.10 In vitro kinase assay

Kinase assays were performed using 25 ng recombinant active p38α protein kinase (Upstate Biotechnology, Charlottesville, USA), 0.2 units/µl recombinant cyclin A-CDK2 and cyclin B-cdc2 (New England BioLabs, UK) in a 40 µl reaction volume (see Appendix) at 30°C for 30 min. Briefly, the appropriate kinase was added to Tbx2-recombinant protein, in the presence of [$\gamma$-$^{32}$P]ATP (see Appendix). Following the kinase reaction, 30 µl of Glutathione Sepharose 4B beads (Amersham Biosciences, USA) equilibrated in kinase buffer, were added to each sample. The samples were washed five times with 1 ml of reaction buffer. Each reaction was terminated by adding 20 µl of 2X SDS loading buffer followed by boiling at 100°C for 10 min. Proteins were resolved on a 12% SDS-PAGE, stained with Coomassie and destained before being dried for autoradiography.

## 2.11 In vitro transcribed translated binding assays

All GST-fusion proteins were expressed in *E.coli* and were purified as described previously (see 2.9). In vitro synthesised proteins were generated from T7 plasmids using the TNT quick coupled transcription-translation system (Promega, USA). One microgram of desired DNA was transcribed and translated at 30°C for 90 min (see Appendix). For the positive control, 2 µl of the reaction mix was added to 3 µl $dH_20$ and 5 µl 2X SDS loading buffer, mixed and heated at 100°C for 10 min. The GST-pulldown assays were performed by incubating equal amounts of GST or GST-fusion protein with Glutathione Sepharose 4B beads (Amersham Biosciences, USA) equilibrated in PBSTi buffer, 0.05 mg/ml bovine serum albumin and 10 µl of in vitro generated [$^{35}$S]-labeled protein. The mixture was incubated at 4°C for 90 min with rolling. Following rolling, the mixture was washed six times in 1 ml PBSTi buffer. After the final wash, the proteins were resuspended in 20 µl 2X SDS loading buffer, boiled for 10 min at 100°C and resolved on a 12% SDS-PAGE. Protein-protein interaction was visualised by autoradiography.

## 2.12 GST-pulldown assays

Cell extracts enriched for cyclin A and cyclin B1 were obtained by lysing cells synchronised in G2 (described in 2.4.2) in RIPA 150 mM NaCl buffer (see Appendix). Cellular debris was removed by centrifugation at 12 000 rpm for 30 min at 4°C. The supernatant of each sample was added to 200 µl of Glutathione Sepharose 4B beads (Amersham Biosciences, USA) equilibrated in RIPA and incubated for 2 hr at 4°C with rolling. Pre-cleared cell lysates were incubated with equal amounts of GST or GST-Tbx2 fusion proteins for 3 hr at 4°C with rolling. Protein-protein

complexes were pelleted by centrifugation at 6 000 rpm for 10 sec at RT and washed five times with 1 ml RIPA buffer. After the final wash, the protein complexes were resuspended in 30 µl 2X SDS loading buffer, boiled for 10 min at 100°C and resolved on a 12% SDS-PAGE for western blot analyses with antibodies to cyclin A (1:1000) and cyclin B1 (1:1000).

## 2.13 Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from MCF-7 cells using the RNeasy Plus Mini kit (Qiagen, USA). The quality and concentration of RNA was determined by spectrophotometry. Only samples exhibiting a $A_{260}/A_{280}$ ratio equal to or above 1.8 were selected and stored at -80°C for further applications. Reverse transcription of RNA was performed in a final volume of 20 µl containing: 4 µl 5X Quantiscript RT buffer, 1 µl Quantiscript Reverse Transcriptase, 1 µl RT Primer mix, 14 µl RNA template (1 µg) (QuantiTect Reverse Transcription kit, Qiagen, USA). The samples were incubated at 42°C for 15 min and the reverse transcriptase inactivated by heating at 95°C for 3 min. Hereafter samples were prepared by real-time PCR. PCR mastermix was prepared as follows: 6 µl RNase-free water, 2 µl 10X QuantiTect primer assay, 10 µl 2X QuantiTect SYBR Green PCR master mix (Qiagen, USA). Eighteen microlitres of PCR mastermix and 2 µl of cDNA were added to glass capillaries. The capillaries were sealed, centrifuged at 3 000 rpm for 30 sec and placed in the LightCycler Version 3 (Roche, Switzerland). PCR cycle parameters were: denaturation (15 min at 95°C), annealing and amplification at 35 cycles (15 sec at 94°C; 20 sec at 55°C; 20 sec at 72°C), melting temperature (15 sec at 65°C) and a cooling step (30 sec at 40°C). Each DNA sample was quantified in duplicate and a negative control without cDNA template was run with every assay to assess the overall specificity. The change in expression was calculated by the $\Delta\Delta Ct$ method (see Appendix). Relative mRNA expression levels were normalised to *GAPDH* for each reaction. Primers used to amplify human *TBX2* (QT00091266) and *p21* (QT00062090) were from Qiagen, USA. GAPDH: forward 5'-GAAGGCTGGGGCTCATTT-3'; reverse 5'-CAGGAGGCATTGCTGATGAT-3'). The Microsoft Excel programme was used to calculate the standard deviation and statistically significant differences between samples using the Student *t* test. *P* values of $<0.05$ were considered statistically relevant.

# CHAPTER 3: RESULTS

As reviewed in section 1.3.2 significant progress has been made in elucidating the signalling pathways that regulate the role of Tbx2 in development. There has also been compelling evidence implicating Tbx2 in the regulation of the cell cycle and altered expression of this gene has been associated with several cancers (see section 1.3.3). However, very little is known about how the levels and function of Tbx2 are regulated during the cell cycle and tumourigenesis. This study has therefore focused on identifying signal transduction pathways and kinases that regulate the activity of Tbx2. The stress-responsive p38 signalling pathway and the mitotic cyclin A/Cdk2 and cyclin B1/Cdk1 were identified as direct regulators of Tbx2. Results are described below.

## 3.1 TBX2 is a target for the stress-responsive DNA damage pathway

### 3.1.1 In vivo phosphorylation of TBX2

In order to determine whether TBX2 is regulated post-translationally by phosphorylation, protein extracted from MCF-7 cells, containing endogenous TBX2, was dephosphorylated with shrimp alkaline phosphatase and subsequently subjected to western blot analyses. Figure 3.1a shows that in contrast to the two bands seen in the untreated sample, only a single intense band was present in the phosphatase treated sample. These results suggest that the top band seen in the untreated sample represents phosphorylated TBX2 and that the protein is phosphorylated in vivo.

When the human and mouse TBX2/Tbx2* sequences were examined for potential phosphorylation sites, using an online motif database (http://au.expasy.org/tools/scanprosite/), thirteen serine-proline (SP) motifs were identified (Fig. 3.1b). Importantly these sites are conserved between the mouse and human proteins with one SP motif located within the T-box (see Fig. 1.1). These motifs are putative target sites for phosphorylation by several kinases, including members of the MAP kinase family (reviewed in Robinson and Cobb, 1997) and the cyclin dependent kinases (Holmes and Solomon, 1996), suggesting that the activity of TBX2/ Tbx2 may be regulated by phosphorylation at these sites. Since TBX2 was previously shown to act as an anti-senescence factor with a role in the cell cycle, we speculated that it may be phosphorylated and regulated by kinases involved in stress-induced signalling pathways and the cell cycle.

### 3.1.2 Transfected Tbx2 is phosphorylated by p38 kinase in response to UVC irradiation

To determine whether Tbx2 is a target for a stress-responsive kinase(s), the phosphorylation status of Tbx2 in response to UVC irradiation was investigated. COS-7 cells,

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* The accepted convention will be used for human (TBX2) and mouse (Tbx2), and when referring to both human and mouse, TBX2/Tbx2 will be used.

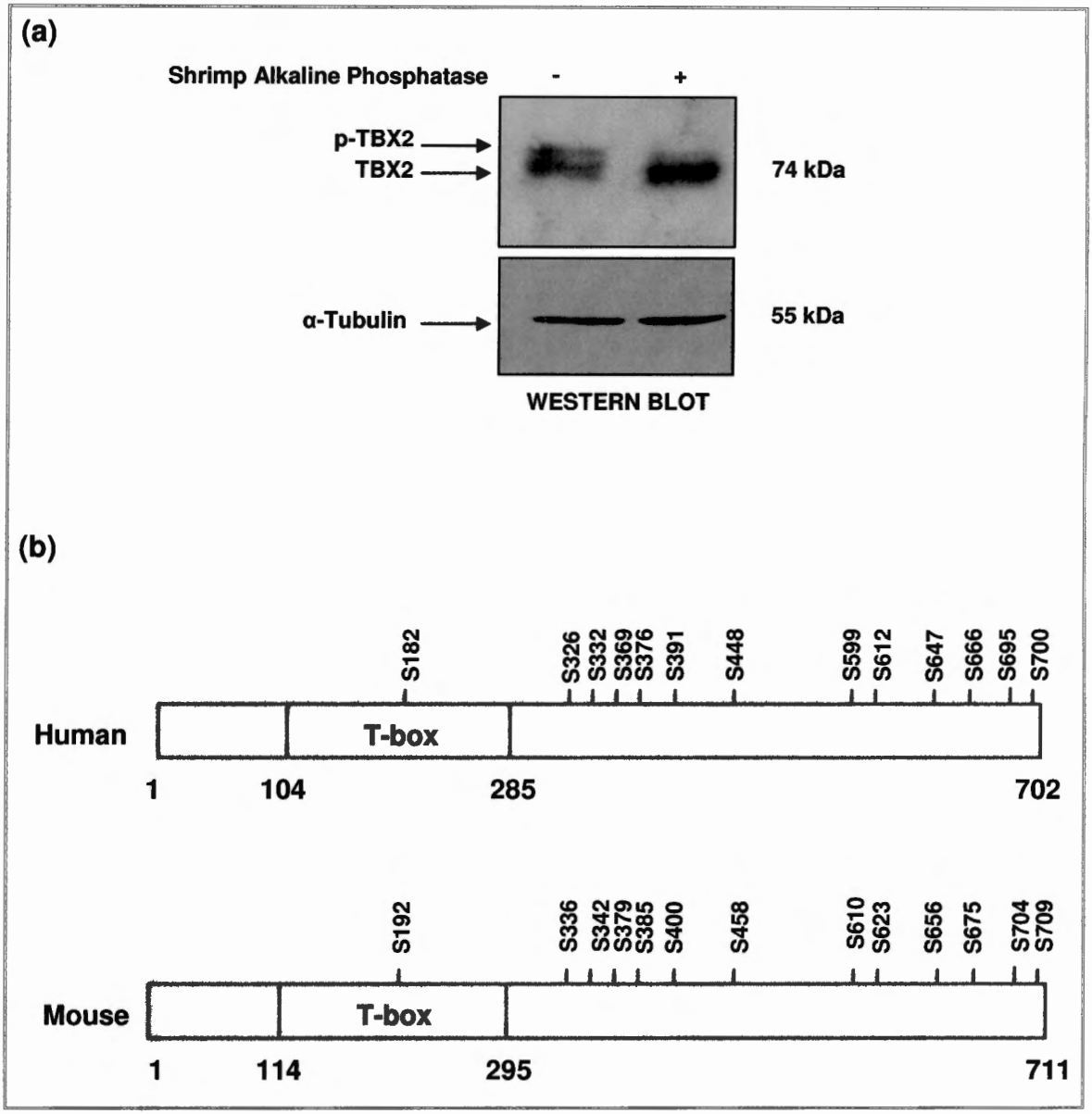


**Figure 3.1.** TBX2 is phosphorylated in vivo. **(a)** Dephosphorylation of TBX2 by shrimp alkaline phosphatase. Protein extracts (30 µg) from MCF-7 cells were dephosphorylated by incubation with 1 unit of shrimp alkaline phosphatase and were separated by 7.5% SDS-PAGE, for optimal separation of phosphorylated TBX2. Western blotting was performed with an antibody to Tbx2 and tubulin was included as a loading control. p–TBX2 is phosphorylated TBX2. **(b)** Schematic representation of the human and mouse Tbx2 protein showing the position of all serine-proline (SP) motifs that are potential phosphorylation sites. The position of the DNA-binding domain, T-box, is shown. The amino acid sequence of human TBX2 (accession no. NP_005985) and mouse Tbx2 (accession no. NP_033350) were obtained from the protein database of the National Centre for Biotechnology Information (NCBI).

that lack endogenous Tbx2, were transfected with a Tbx2 expression vector, exposed to increasing doses of UVC irradiation and protein extracts analysed by western blotting. Figure 3.2a shows that with increasing doses of UVC irradiation there is an increase in phosphorylated Tbx2 from 10 J/m$^2$. This observed increase in phosphorylated Tbx2 is consistent with the appearance of activated p38, as detected using an antibody specific for phosphorylated p38. These results were confirmed by densitometric scanning of autoradiographs and the results are represented in the bar graph shown in the lower panel of Figure 3.2a.

In response to a variety of cellular stresses, including UV irradiation, the JNK and p38 MAP kinase are strongly activated (reviewed in Kyriakis and Avruch, 2001). To identify which of these two kinases are responsible for Tbx2 phosphorylation, the above experiment was repeated in the presence of SB203580, which specifically inhibits p38, while being ineffective against JNK. In this experiment transfected COS-7 cells were exposed to a dose of 40 J/m$^2$ UVC because it consistently yielded high levels of phosphorylated Tbx2 in many different experiments. Tbx2 was detected as a single band (Fig. 3.2b) because the protein samples were analysed on a 10% SDS-PAGE in this experiment, which does not allow resolution of phosphorylated Tbx2. On close inspection it is apparent that the band in the UVC irradiated cells migrates higher than that in the mock irradiated cells confirming that transfected Tbx2 is phosphorylated in cells exposed to UVC. In the presence of SB203580, however, the UVC-dependent phosphorylation of Tbx2 is partially inhibited as the protein migrates higher than the mock irradiated but lower than the UVC irradiated samples. A possible explanation for this partial inhibition is that the inhibitor did not completely abolish the activity of the p38 kinase since a faint signal was obtained for the active form of p38. Furthermore, activation of the p38 pathway was confirmed by the presence of phosphorylated p38 in the UVC irradiated cells only, which was inhibited by SB203580. These results showed that the transfected Tbx2 protein was phosphorylated by the stress-responsive p38 kinase in response to UVC irradiation.

### 3.1.3 Endogenous TBX2 is phosphorylated by p38 kinase in response to UVC irradiation

Since the above experiments were done on cells expressing exogenously introduced Tbx2, it was important to determine whether UVC had the same effect on endogenous Tbx2. MCF-7 breast cancer cells that overexpress TBX2 were thus exposed to a fixed dose of 100 J/m$^2$ UVC, and protein harvested at various time points post-treatment was analysed by western blotting. The dose of UVC selected in this experiment was based on previous results, which showed that it consistently yielded high levels of phosphorylated TBX2 in MCF-7 cells. Figure 3.3a shows an increase in the phosphorylation status of TBX2 immediately after UVC irradiation (0 min) with high levels persisting up to 180 min post-treatment. It is interesting to note that the lower TBX2 band is also more intense immediately post-UVC treatment and is markedly elevated by 10 min. This would suggest that the effect seen on the lower band at these early time points is also due to post-translational modifications, such as acetylation, rather than due to increased mRNA levels. Activation of the p38 pathway was only observed in the UVC treated cells, while total p38 indicated equal loading in all lanes.

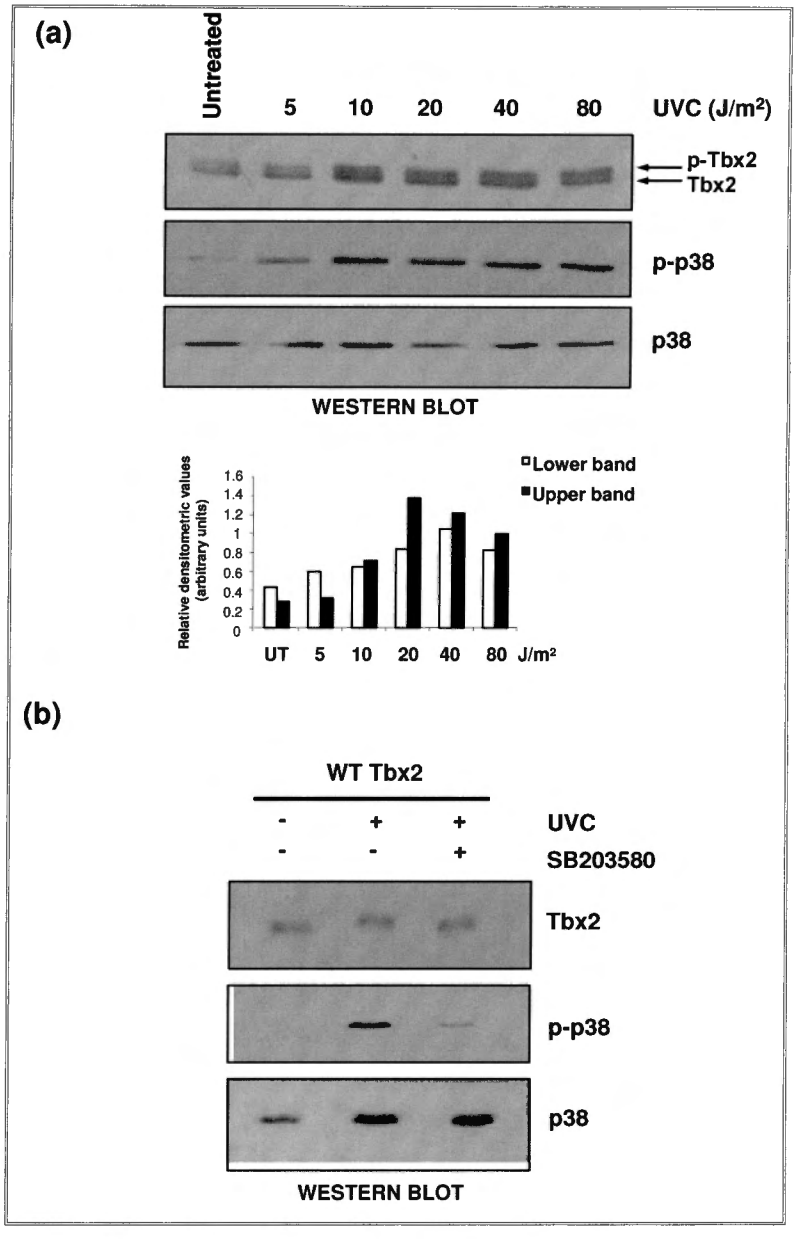


**Figure 3.2.** UVC-induced phosphorylation of transfected Tbx2 by p38 kinase. **(a)** Dose-dependent increase in the phosphorylation status of transfected Tbx2 in response to UVC irradiation. COS-7 cells were transfected with a vector expressing SV5 epitope-tagged Tbx2 and UVC irradiated at the indicated dosages. Protein extracts (3 µg) were prepared 60 min post treatment. In order to detect phosphorylated Tbx2, protein samples were analysed on a 7.5% SDS-PAGE and by western blotting using an antibody to SV5. Levels of phospho-p38 and total p38 in these samples were assessed on a 12% SDS-PAGE and by western blotting. The bar graph compares the intensity of the upper (p-Tbx2) and lower (Tbx2) bands to the p38 loading control for each sample. **(b)** Stress-induced phosphorylation of Tbx2 is inhibited in the presence of the p38 inhibitor. COS-7 cells were pretreated with 10 µM SB203580, 30 min prior to 40 J/m² UVC irradiation. To accurately detect Tbx2 as a single band an equal amount of protein from each sample was analysed by 10% SDS-PAGE with an antibody to SV5. Levels of phospho-p38 and total p38 were detected using the appropriate antibodies.

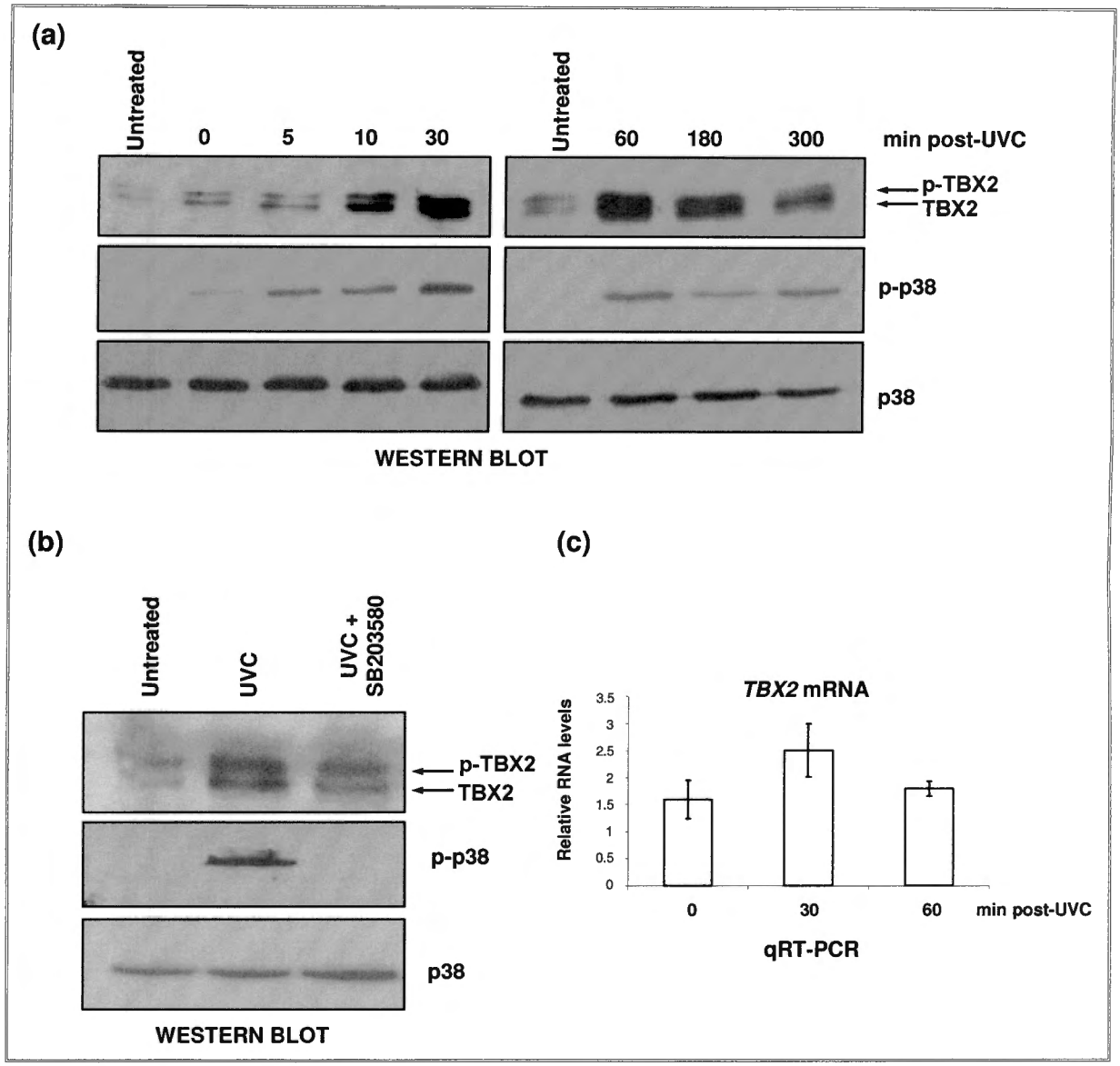


**Figure 3.3.** Endogenous TBX2 is phosphorylated in response to UVC by p38 kinase. **(a)** Time-dependent increase in the phosphorylation of endogenous TBX2 in response to UVC irradiation. Protein extracts (44 µg) from MCF-7 cells at the indicated time points post-UVC irradiation (100 J/m$^2$) were separated by 7.5% SDS-PAGE and by western blotting to detect phosphorylated TBX2. Levels of phospho-p38 and total p38 in these samples were assessed on a 12% SDS-PAGE and by western blotting with appropriate antibodies. The protein extracted from 0 to 30 minutes and 60 to 300 minutes post-UVC treatment were from different experiments and were therefore analysed on separate gels. **(b)** Endogenous TBX2 is phosphorylated in vivo by p38 kinase. MCF-7 cells irradiated with UVC (100 J/m$^2$) in the presence or absence of SB203580 (10 µM, 30 min prior to UVC irradiation). Protein extracts (63 µg) were assayed as described in (a) above. **(c)** *TBX2* mRNA levels increase in response to UVC irradiation. Quantitative real-time PCR of endogenous *TBX2* mRNA extracted from MCF-7 cells at the indicated times post-UVC irradiation (100 J/m$^2$). The levels of *TBX2* mRNA expression were normalised against *GAPDH*. Error bars represent means ± standard deviation.

To confirm that this increase in phosphorylated TBX2 is due to activation of the p38 kinase, MCF-7 cells were exposed to UVC irradiation (100 J/m$^2$) in the presence of SB203580. Based on the data represented in Fig. 3.3a, protein was harvested 60 min post-UVC irradiation and analysed by western blotting. Figure 3.3b shows that the UVC induced increase in endogenous TBX2 phosphorylation occurs specifically via the p38 MAP kinase as treatment with the p38 inhibitor significantly reduces this effect.

To determine the effect of UVC irradiation on *TBX2* mRNA levels, quantitative real-time PCR was performed. RNA, extracted from MCF-7 cells at 0, 30 and 60 minutes post-UVC irradiation, was amplified using primers specific for *TBX2*. The *TBX2* mRNA expression levels were normalised against the house keeping gene, *GAPDH*. Figure 3.3c shows that *TBX2* mRNA levels increased immediately post-UVC irradiation, peaking at 30 min. The mechanism by which UVC irradiation leads to an increase in *TBX2* mRNA levels is discussed in Chapter 4. Taken together, the results of the above experiments suggest that TBX2 protein levels increase in response to UVC irradiation due to both a phosphorylation dependent increase in the stability of the protein and an increase in mRNA levels.

### 3.1.4 Tbx2 is phosphorylated at S336, S623 and S675 by p38 kinase in vitro

Having established that TBX2/Tbx2 is phosphorylated by p38 kinase in vivo, the next step was to identify these p38 target sites. SP and TP motifs are the minimum potential target sites for phosphorylation by the p38 kinase. The serine residues in the 13 candidate SP motifs within the Tbx2 protein were therefore individually mutated to alanine by site-directed mutagenesis (these were generated by Dr S. Prince) and tested in in vitro kinase assays. Briefly, the wild-type (WT)- and mutant Tbx2 proteins were expressed as GST fusion proteins and used as substrates for the p38$\alpha$ kinase in the presence of [$\gamma$-$^{32}$P]ATP and the results visualised by autoradiography. In these kinase assays the GST-Tbx2 fusion proteins were expressed as either N-terminal (1-371) or C-terminal (371-711) proteins (Fig. 3.4a) as the full-length GST-Tbx2 protein was found to be very unstable in these assays.

The results (Fig. 3.4b) indicate that both the WT N- and C-terminal Tbx2 proteins were phosphorylated by the p38 kinase, which suggested that there were p38 target sites within both these portions of the protein. There are three SP motifs in the N-terminal Tbx2 protein and the S192A and S342A mutants had no effect on phosphorylation. The S336A mutation however abolished phosphorylation of the N-terminal Tbx2 protein suggesting that it was the only p38 target site in this part of the protein. In this experiment the S336A:S342A double mutant was included and results show that it behaved as the S336A mutant. The single mutations S623A and S675A reduced phosphorylation of the C-terminus of Tbx2 and when both these sites were mutated, phosphorylation was considerably reduced. Mutating the remaining eight potential p38 target sites in the C-terminus of Tbx2 had no effect on phosphorylation of the protein (data not shown). GST alone was used as a negative control. The lower panels in Figure 3.4b show the same gels stained with Coomassie Blue indicating that equivalent amounts of GST-fusion proteins were used in these experiments. The bar graphs show densitometric values of radioactive levels measured for each construct used in the kinase assay. The results from these

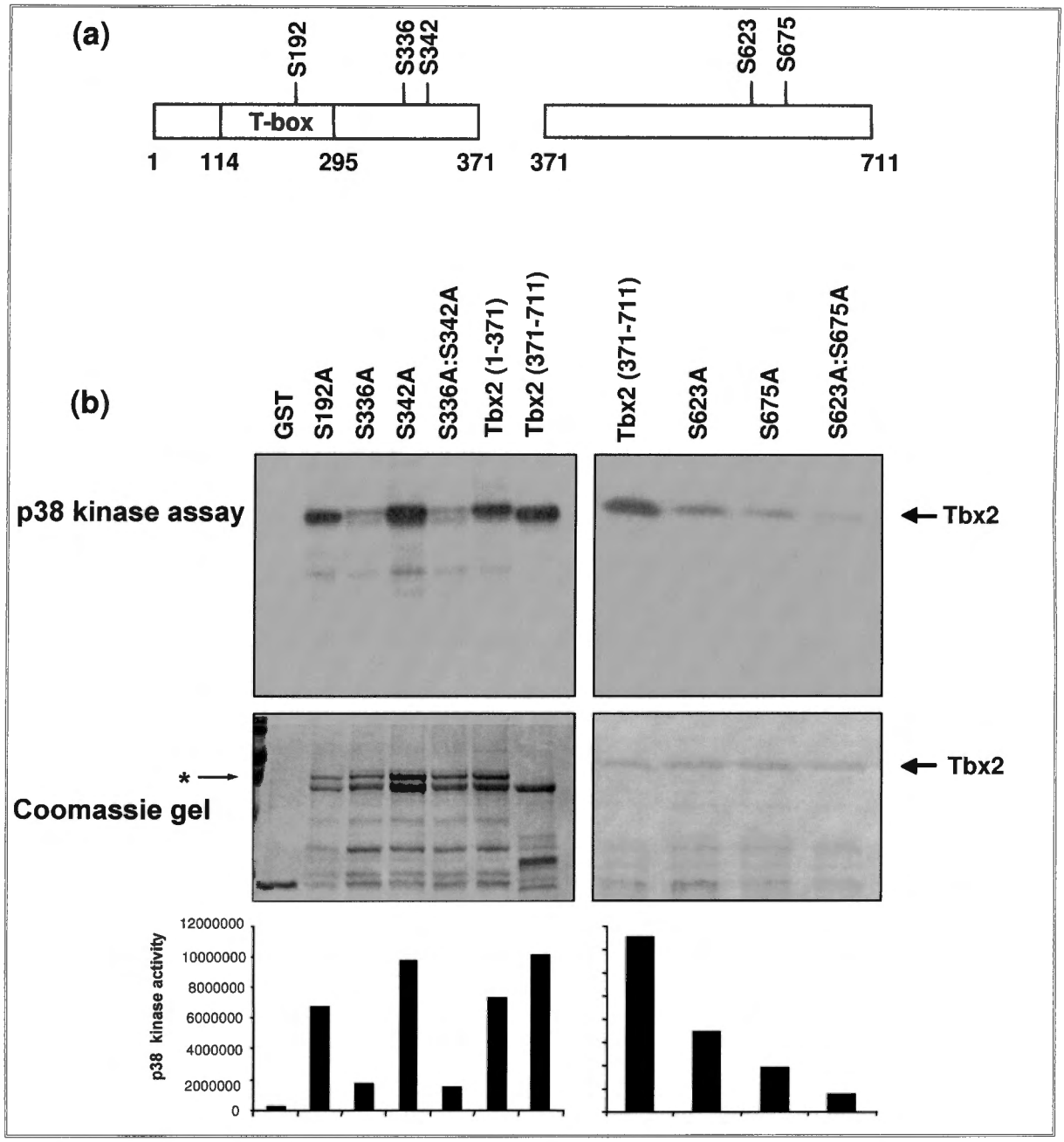


**Figure 3.4.** Tbx2 is phosphorylated by p38 kinase at S336, S623 and S675 in vitro. **(a)** Schematic representation of the N-terminal (1-371) and C-terminal (371-711) Tbx2 proteins used as substrates in p38 kinase assays. The sites mutated in constructs used in the kinase assays shown in (b) are indicated. **(b)** Mapping the p38 kinase target sites within Tbx2. In vitro p38 kinase assays were performed using purified GST-Tbx2 fusion proteins as substrates in the presence of the recombinant activated p38α kinase and [$\gamma$-$^{32}$P]ATP. The mutant GST-Tbx2 fusion proteins were generated by site-directed mutagenesis as described in section 2.2 of the material and methods. Kinase assays using the indicated Tbx2 proteins are shown in the upper panel after 12% SDS-PAGE and autoradiography. The middle panel shows the same gels stained with Coomassie Blue indicating that equivalent amounts of protein were used in each assay. The bar graphs show densitometric values of radioactive levels measured for each construct in the kinase assay. (*) Refers to the 1-361 truncated Tbx2 protein.

assays identified S336, S623 and S675 as sites in the Tbx2 protein that were phosphorylated by the p38 kinase in vitro.

### 3.1.5 Tbx2 is phosphorylated at S336, S623 and S675 by p38 kinase in vivo response to UVC irradiation

To determine whether the p38 target sites identified in the in vitro kinase assays are phosphorylated in vivo, a mutant was generated in which all three serine residues were mutated to alanine in the full-length Tbx2 protein (Tbx2 S336A:S623A:S675A). COS-7 cells were transfected with vectors expressing either SV5-tagged WT Tbx2 or a SV5-tagged Tbx2 S336A:S623A:S675A mutant and their phosphorylation status compared by western blot analyses. Figure 3.5a shows that unlike WT Tbx2, the Tbx2 mutant is not phosphorylated and is expressed as a single band. These results suggest that serine residues 336, 623 and 675 are indeed targets for in vivo phosphorylation. Given that mutating these three serine residues results in the complete loss of the upper phosphorylated band, would imply that they are critical for phosphorylation by an additional kinase(s).

The next experiment investigated whether the identified serine residues were the only p38 kinase target sites in response to UVC irradiation. To this end, cells were transfected with vectors expressing either SV5-tagged WT Tbx2 or the SV5-tagged Tbx2 (S336A:S623A:S675A) mutant in the presence or absence of UVC and the Tbx2 phosphorylation status was compared (Fig. 3.5b). In the untreated cells transfected with WT Tbx2, two bands were obtained although the intensity of the top band was much weaker in this experiment. As expected, the intensity of both WT Tbx2 bands increased in response to UVC irradiation. Mutating the serine residues 336, 623 and 675, however, abolished this effect since only a single band was observed in both treated and untreated cells. Activation of the p38 pathway was only observed in the UVC treated cells and total p38 showed equal loading. These results confirmed that the identified sites were the only p38 targets for phosphorylation of Tbx2 in response to UVC.

### 3.1.6 The TBX2 protein is regulated by p38 kinase phosphorylation

Having established that TBX2 is phosphorylated by the p38 kinase in response to UVC-induced cellular stress, the question arose as to whether phosphorylation might regulate its protein stability and subcellular localisation?

#### 3.1.6.1 Pseudo-phosphorylation of the p38 target sites prevents Tbx2 degradation

Previous results (see Fig. 3.3a) showed an increase in phosphorylated TBX2 immediately post-UVC treatment (0 min), which suggested that the stability of the protein was regulated by phosphorylation. To address this possibility the effect of p38 phosphorylation on Tbx2 protein stability was investigated. This was achieved by mutating the p38 target sites to alanine (A), to abolish phosphorylation or to glutamic acid (E), to mimic phosphorylation. NIH 3T3 cells, transiently transfected with either WT Tbx2 or the Tbx2 mutants, were incubated with cycloheximide over a period of 6 hours and protein extracts were subjected to western blot analyses (Fig. 3.6). In order to accurately quantify total levels of Tbx2 in this experiment, cell lysates were separated on a 10% SDS-PAGE and hence Tbx2 is detected as a single band. The

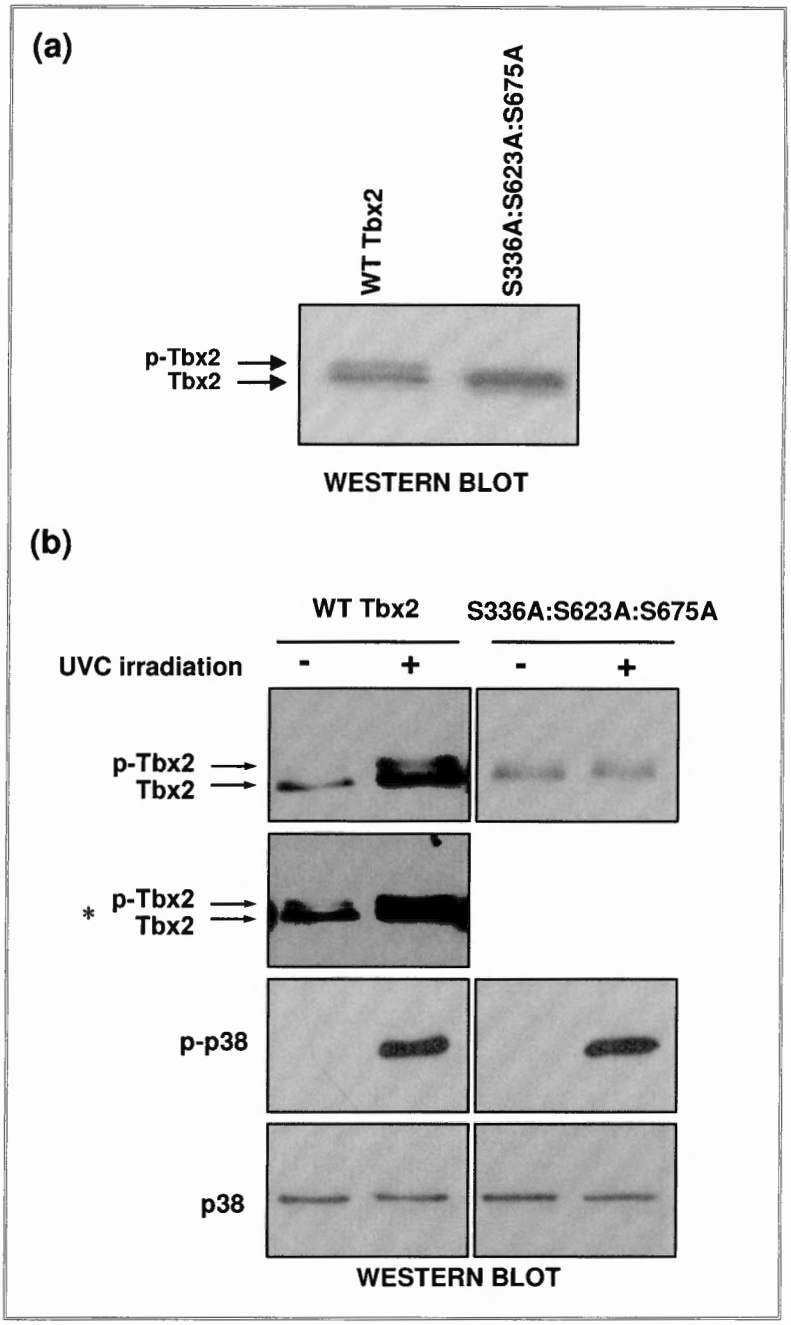


**Figure 3.5.** Tbx2 is phosphorylated at S336, S623 and S675 in vivo. **(a)** Mutating the p38 kinase sites affects the phosphorylation of Tbx2 in vivo. SV5 epitope-tagged WT Tbx2 or the SV5 epitope-tagged Tbx2 S336A:S623A:S675A mutant were expressed in COS-7 cells and an equal amount of protein from each sample was separated by 7.5% SDS-PAGE and by western blotting to analyse the phosphorylation status of Tbx2 using an anti-SV5 antibody. p–Tbx2 is phosphorylated. **(b)** The serine residues 336, 623 and 675 in the Tbx2 protein are the only sites phosphorylated by p38 in response to UVC. Cells were transfected with the Tbx2 constructs described in (a) and exposed to UVC (40 J/m$^2$) 29 hrs post-transfection. Protein extracts were prepared 60 min post-UVC treatment and Tbx2 was detected as described in (a). Phospho-p38 and total p38 were run on a 12% SDS-PAGE and assessed by western blotting using the appropriate antibodies. (*) Indicates a longer exposure of the blot.

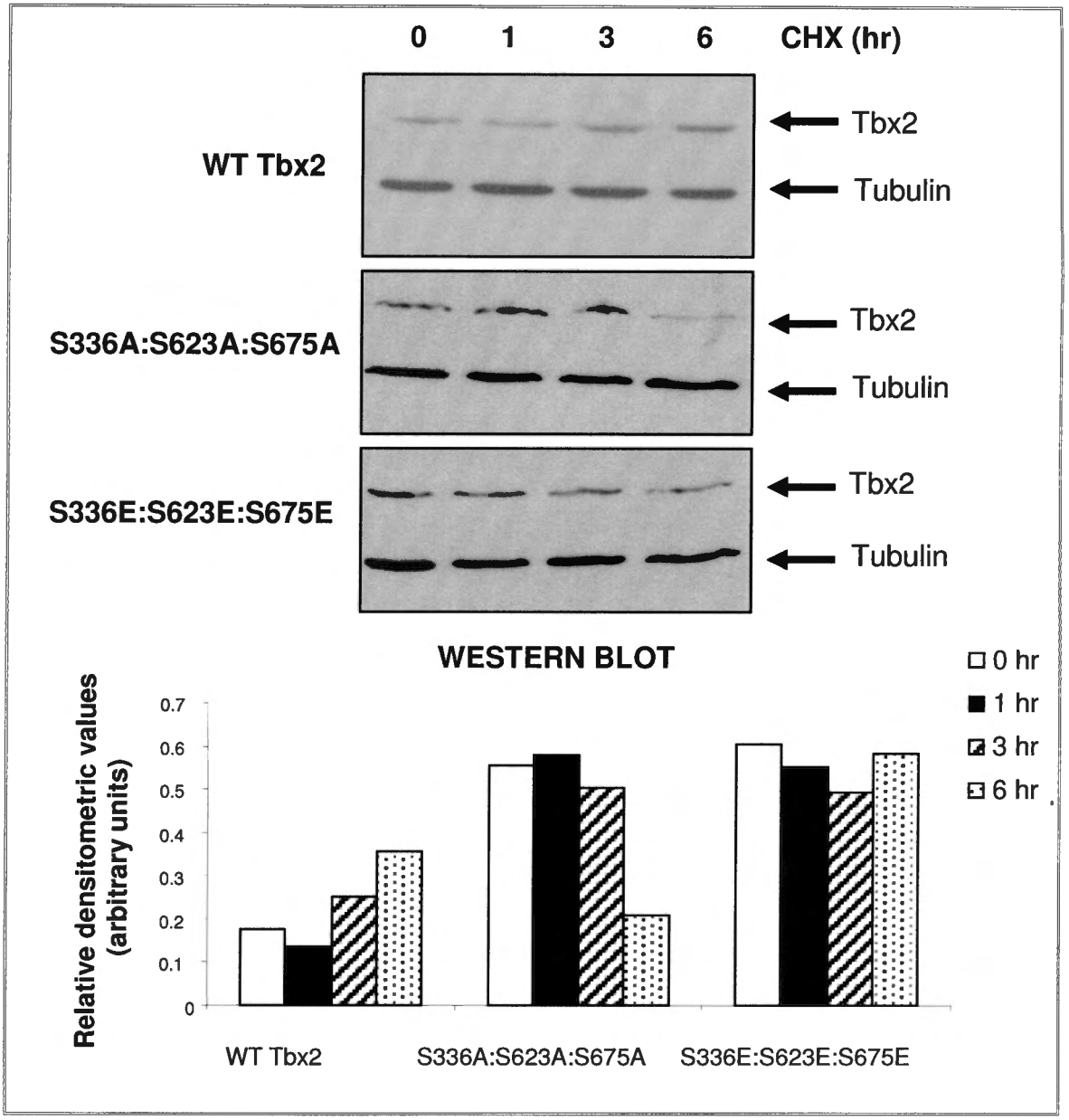


**Figure 3.6.** Pseudo-phosphorylation at the p38 target sites increases Tbx2 protein stability. NIH 3T3 cells transiently transfected with vectors expressing SV5 epitope-tagged Tbx2 proteins as indicated, were incubated 48 hr post-transfection with 30 µg/ml cycloheximide (CHX) for the indicated times. To accurately detect total levels of the Tbx2 protein as a single band, an equal amount of protein from each sample was analysed on a 10% SDS-PAGE and by western blotting with anti-SV5 antibodies. The bar graph compares the intensity of Tbx2 normalised to the tubulin loading control.

Tbx2 S336A:S623A:S675A mutant protein was unstable with an approximate 30% decrease in protein levels at 6 hours. In contrast, the levels of the pseudo-phosphorylated Tbx2 S336E: S623E:S675E mutant remained unchanged throughout the treatment. In this experiment the WT Tbx2 protein increases slightly over the period of cycloheximide treatment and it is not clear whether this is of any significance as, in similar experiments, the protein mimics the levels of the pseudo-phosphorylated protein. The densitometric graph in the lower panel compares the intensity of the Tbx2 protein normalised to the tubulin loading control. This finding implicates the p38 kinase as a modulator of Tbx2 protein stability in response to cellular stress.

It is important to note that in these experiments, NIH 3T3 fibroblasts were used because, compared to COS-7 cells they transfect less efficiently (20% vs 90%) and thus increase the chance of distinguishing small changes in the levels of transfected protein. Furthermore, the high transfection efficiency seen in COS-7 cells was expected to lead to the expression of high levels of the transfected protein, which may compromise the proteolytic machinery.

#### 3.1.6.2 UVC-induced phosphorylation of TBX2 by p38 kinase induces nuclear translocation of TBX2

In the course of this study TBX2 was observed to be both cytoplasmic and nuclear in MCF-7 cells by immunofluorescence. To establish whether phosphorylation by the p38 MAP kinase affected the subcellular localisation of TBX2, MCF-7 cells were mock irradiated (untreated) or UVC-irradiated in the presence or absence of the p38 inhibitor, SB203580. The cells were processed for immunofluorescence with an antibody to Tbx2 (Fig. 3.7). As expected, TBX2 was found to exhibit diffuse staining both in the cytoplasm and nucleus of the untreated cells. In UVC-irradiated cells, however, TBX2 was predominantly localised in the nuclei and cells pre-treated with the p38 inhibitor have a similar staining pattern to that seen in untreated cells. These results are emphasised at high magnification (Fig. 3.7, lower panel) and indicate that phosphorylation of TBX2 by the p38 kinase in response to UVC irradiation results in the almost exclusive localisation of the protein to the nucleus.

Taken together the above results showed that phosphorylation of TBX2 by the p38 kinase in response to UV-induced stress regulates TBX2 protein stability and subcellular localisation.

### 3.1.7 UVC-induced phosphorylation by p38 enhances the ability of Tbx2 to repress *p21*

The above results suggested that TBX2 may be playing a role in the UV stress pathway. Interestingly, it is well established that p21, a known Tbx2 target (Prince et al., 2004), is critical for mediating the cell cycle arrest required for DNA damage repair post-UV irradiation (reviewed in Fotedar et al., 2004). However, recent reports suggest that p21 inhibits DNA repair (Pan et al., 1995; Cooper et al., 1999; Bendjennat et al., 2003) and that subsequent to establishing a cell cycle arrest following UV irradiation, p21 has to be downregulated (McKay et al., 1998; Wang et al., 1999; Bendjennat et al., 2003). This UV-mediated downregulation of p21 was previously observed at both the protein and mRNA levels in MCF-7 cells (Wang et al., 1999) and it was hypothesised that TBX2 may be required for the decrease in *p21* mRNA

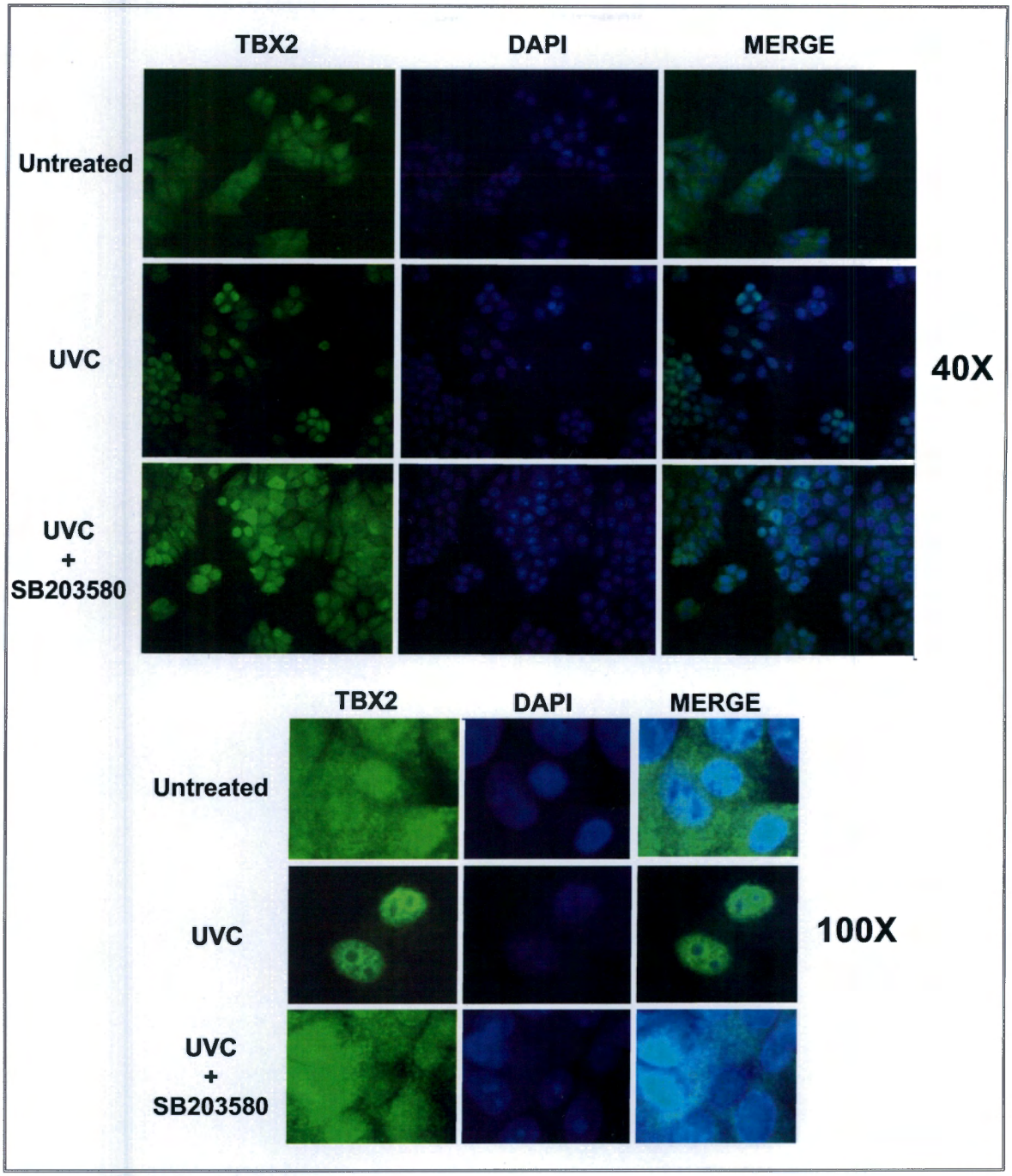


**Figure 3.7.** p38 phosphorylation induces nuclear translocation of TBX2. MCF-7 cells were irradiated with UVC (100 J/m$^2$) in the presence or absence of SB203580 and then analysed 60 min post-treatment by immunofluorescence using an antibody to Tbx2. All cells were stained with DAPI, to determine the location of the nuclei. The upper and lower panels represent images captured at 40X and 100X magnifications, respectively.

levels. This study tests this hypothesis by firstly investigating whether there was an inverse correlation between TBX2 and p21 protein levels in UVC irradiated MCF-7 cells (Fig. 3.8a). The results show that immediately post-UVC irradiation (0 min) there was a substantial increase in p21 levels followed by a decrease, which corresponded with increased TBX2 levels at all subsequent time points. It is important to point out that the protein extracted from the 0 to 30 minutes and 60 to 300 minutes post-UVC treatment were from separate experiments and were therefore analysed on different gels. This may account for the difference in p21 protein levels seen in the untreated lanes of the two blots and thus does not detract from the interpretation of the results. Levels of p-p38 are included to show activation of the p38 pathway.

To determine whether the decrease in p21 seen above was due to reduced levels of *p21* mRNA, quantitative real-time PCR was performed. Results (Fig. 3.8b) revealed that relative to the untreated cells *p21* mRNA levels were downregulated immediately after UVC treatment and remained low over the period when TBX2 protein levels went up. The results of the above western blot and quantitative real-time PCR experiments thus reveal an inverse correlation between TBX2 protein and *p21* mRNA levels in UVC irradiated MCF-7 cells. This suggests that TBX2 may be involved in the reduced levels of *p21* seen under these conditions.

The possibility that stress-induced phosphorylation by p38 kinase potentiates the ability of Tbx2 to repress transcription of the *p21* gene, was next investigated. COS-7 cells were co-transfected with a WT Tbx2 expression vector together with a *p21* promoter-luciferase (LUC) reporter and the cells treated with or without UVC irradiation. Basal promoter activity was determined using the empty control vector (pCMV19.SV5). The results of three independent experiments in which each construct was tested in duplicate, are shown in Figure 3.9a. Consistent with our previously published data (Prince et al., 2004), Tbx2 repressed the *p21* promoter by approximately 2 fold. In the presence of UVC irradiation, however, repression of the *p21* promoter by Tbx2 was significantly enhanced by approximately 5 fold. TBX2 has previously been shown to repress the $p14^{ARF}$ gene (Jacobs et al., 2000; Lingbeek et al., 2002) and therefore the effect of UVC on repression of this gene was next tested. Figure 3.9b shows that while Tbx2 repressed the $p14^{ARF}$ promoter by approximately 6 fold, this repression was only marginally enhanced by UVC treatment. These results suggest that the enhancement of Tbx2 mediated repression by UVC is specific to the *p21* promoter.

The next experiment tested whether the observed repression of *p21* by Tbx2 was mediated through phosphorylation of Tbx2 by the p38 kinase. A comparison of the ability of WT Tbx2 to repress the *p21* promoter, to that of the Tbx2 S336A:S623A:S675A and S336E:S623E:S675E mutants were thus performed. The results in Figure 3.9c indicate that whereas the WT and pseudo-phosphorylated Tbx2 mutant repressed the *p21* promoter, abolishing the p38 target sites (A mutant) led to an abrogation of this repression. The reason why the pseudo-phosphorylated Tbx2 protein behaves as the WT Tbx2 is unclear but it is possible that substitution of serine residues with glutamic acid does not mimic exactly the effect of phosphorylation at these sites. Another possible explanation may be that WT Tbx2 is phosphorylated by p38 kinase due to the transfection process which is thought to generate a stress response (personal communication

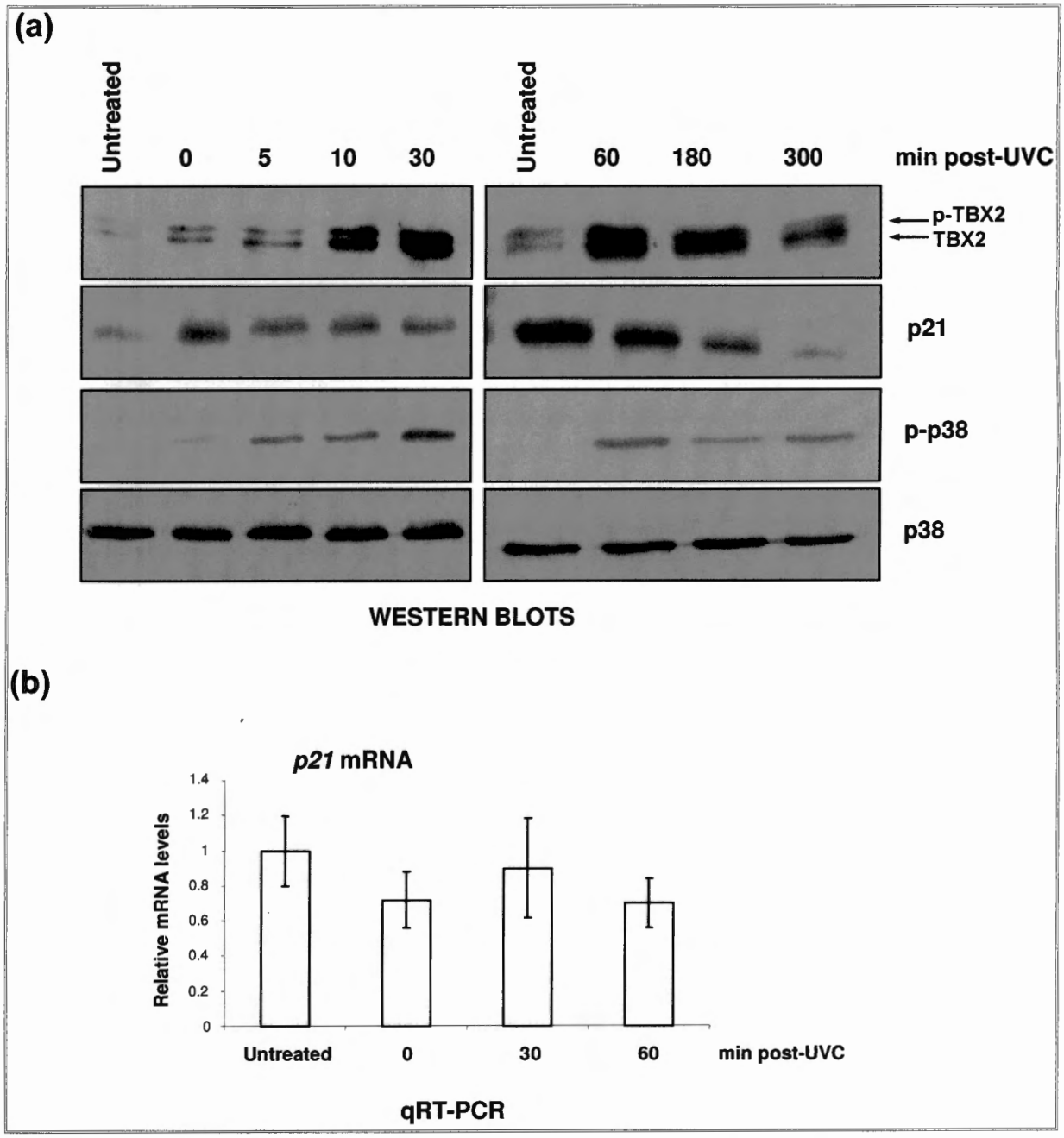


**Figure 3.8.** Inverse correlation between TBX2 and p21 protein and mRNA levels in response to UVC irradiation. **(a)** p21 protein levels decrease post-UVC treatment. Protein extracts (the same used in Fig. 3.3a) from MCF-7 cells at the indicated time points post-UVC irradiation (100 J/m$^2$) were analysed by 15% SDS-PAGE and western blotting using an antibody to p21. The protein extracted from 0 to 30 minutes and 60 to 300 minutes post UVC-treatment were analysed on different gels. The results for TBX2, p-p38 and total p38 are the same as shown in Fig. 3.3a. **(b)** *p21* mRNA levels are repressed in response to UVC irradiation. Total RNA was extracted from MCF-7 cells at the times indicated post-UVC irradiation. Quantitative real-time PCR was then performed on reverse transcribed RNA using primers specific to *p21* and mRNA levels were normalised to *GAPDH*. Error bars represent means ± standard deviation.

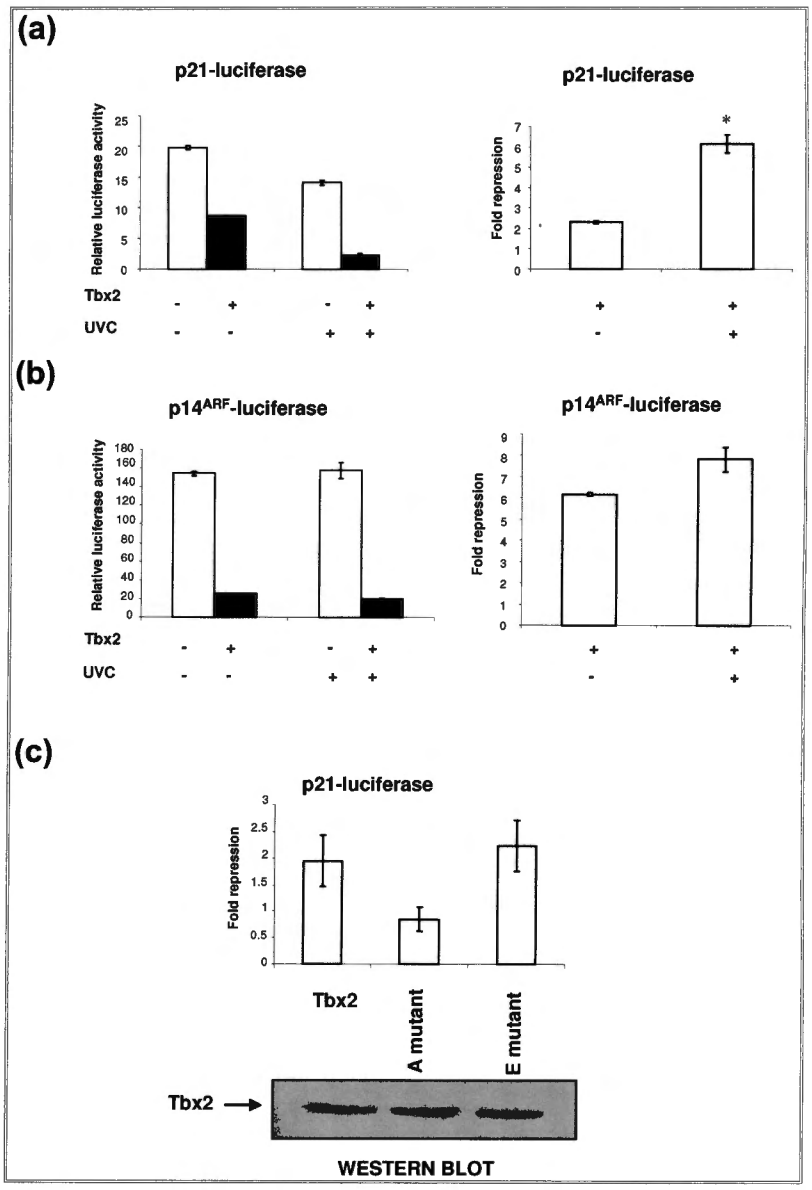


**Figure 3.9.** UVC-induced phosphorylation by p38 kinase enhances the ability of Tbx2 to repress *p21*. **(a)** UVC irradiation enhances the transcriptional repression of *p21* by Tbx2. The *p21* promoter-luciferase reporter (700 ng) was co-transfected into COS-7 cells either with the empty pCMV (200 ng) vector or a Tbx2 (200 ng) expression vector, the cells treated with and without UVC (40 J/m$^2$) and luciferase activity determined (left panel). The right panel depicts the fold repression which represents the ratio of the luciferase activity generated by the pCMV empty vector (without Tbx2) to that obtained in the presence of pCMV-Tbx2. (*) Indicates significant difference from the control (no UVC treatment) at $P<0.05$. **(b)** Effect of UVC irradiation on the ability of Tbx2 to transcriptionally repress *p14$^{ARF}$*. The experiment was repeated under the same conditions as described in (a) using 700 ng of the *p14$^{ARF}$* promoter-luciferase reporter. **(c)** Mutating the p38 target sites abolishes the ability of Tbx2 to transcriptionally repress the *p21* promoter. COS-7 cells were transfected as described in (a) with the Tbx2 expression vectors, were the identified p38 target sites are either mutated to abolish phosphorylation (A mutant) or pseudo-phosphorylated (E mutant) as indicated and luciferase activity determined. Western blotting shows equal expression of WT Tbx2 and the Tbx2 mutants in the luciferase assays. Error bars represent means ± standard deviation.

Dr M Galibert). To exclude the possibility that the results obtained may be due to different levels of Tbx2 expression for the different constructs used in the transfection assay, protein was harvested from parallel dishes and subjected to western blot analyses. The results show that equal protein expression was observed for each of the constructs (Fig. 3.9c, lower panel). These results suggest that the identified p38 target sites do indeed play a role in regulating the transcriptional activity of Tbx2 on the *p21* promoter in response to UVC irradiation.

## 3.2 The regulation and role of TBX2/Tbx2 in the cell cycle

Several lines of evidence have implicated a role for TBX2 in the cell cycle (see Chapter 1). Furthermore, data generated in our laboratory and that of our collaborator, Dr C. Goding (Marie Curie Research Institute, UK), have shown that TBX2 binds mitotic chromatin, nucleosomal DNA, interacts with the histone H3 tail (Demay et al., 2007) and represses the cyclin-dependent kinase inhibitor, *p21* (Prince et al., 2004). Based on these data it was speculated that TBX2 protein expression would be tightly regulated during the cell cycle. A recent study by Bilican and Goding (2006) in the B16 mouse melanoma cell line showed that Tbx2 levels are low in G1, increase in mid-S-phase and persist at high levels through G2 until finally undergoing a dramatic reduction at the onset of mitosis. This change in Tbx2 protein levels does not correspond to changes in *Tbx2* mRNA levels, suggesting that the protein may be regulated by post-translational modifications such as phosphorylation. This possibility was thus explored in the present study.

### 3.2.1 Cell cycle dependent regulation of the phosphorylation status of Tbx2

In order to determine whether levels of Tbx2 during the cell cycle are regulated by phosphorylation, conditions for synchronising cells in the various phases of the cell cycle had to be established. This was accomplished by chemically synchronising B16 cells at specific phases of the cell cycle and analysing the cells by flow cytometry. The optimised conditions were as follows: cells were synchronised by a double thymidine block to obtain a G1/S population and a S-phase population was obtained using the same conditions followed by a 2 hr release from the thymidine block. G2/M cells were obtained by a thymidine block followed by nocodazole treatment, at the end of which mitotic cells were separated by shake-off and the adherent cells taken as a G2 population. The cell cycle phase distribution was assessed by measuring DNA content using flow cytometry. The data shown in Figure 3.10a confirmed that the cells were indeed synchronised in the appropriate phases and these conditions were therefore used for future experiments using the B16 cell line.

To investigate the phosphorylation status of the endogenous Tbx2 protein at the various phases of the cell cycle, synchronised cells were collected and protein extracts were analysed by western blotting. The results shown in Figure 3.10b indicate that whereas levels of phosphorylated Tbx2 are equal in G1 and S-phase cells, they are substantially elevated in G2 cells and dramatically reduced in mitosis. These results confirmed that the level of phosphorylated Tbx2 is regulated during cell cycle progression. It is worth noting that the intensity of the lower band also increased at G2. Furthermore, these results suggested that Tbx2 may be playing a role in G2 and that

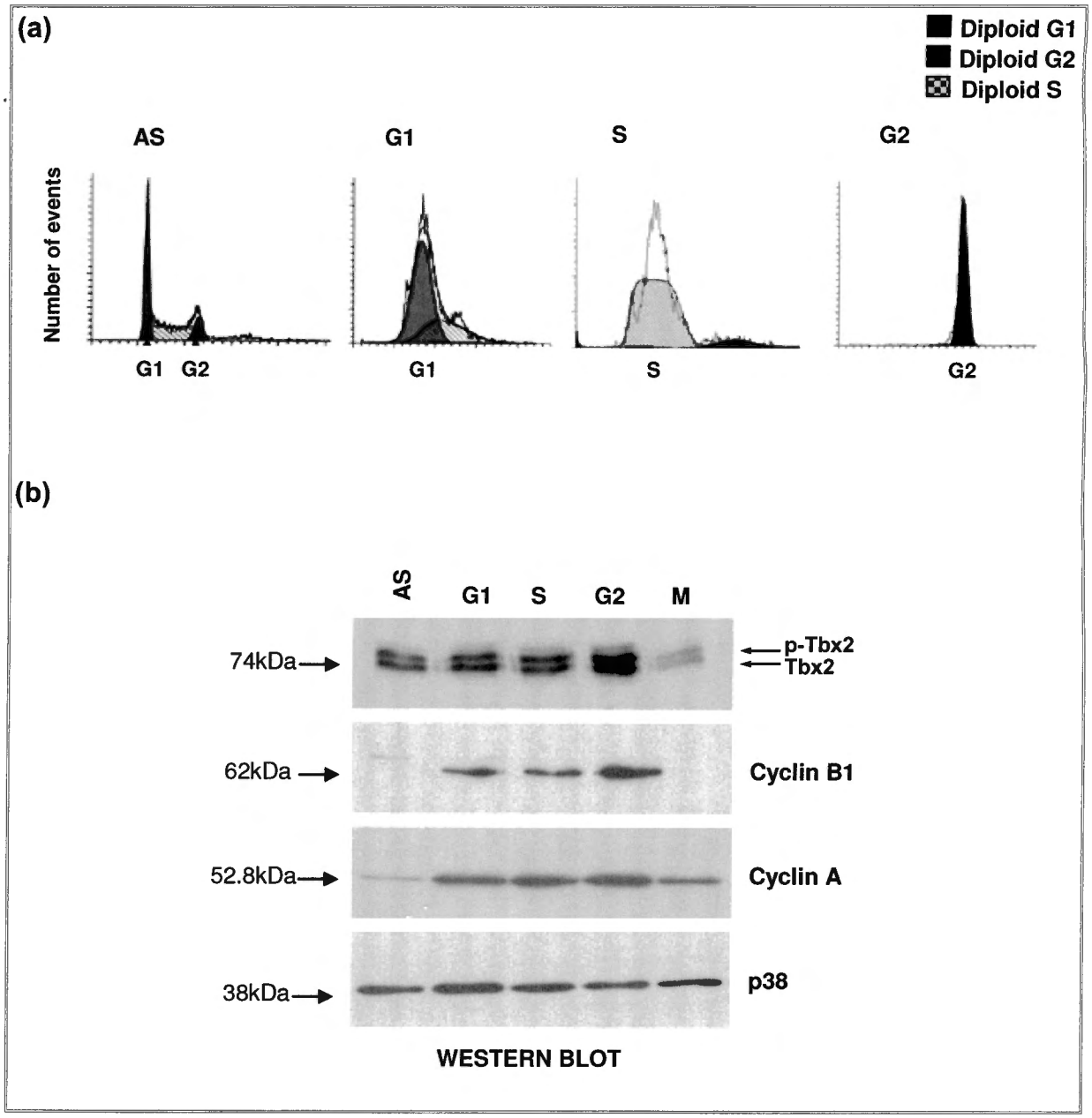


**Figure 3.10.** The phosphorylation status of Tbx2 is regulated during the cell cycle with levels peaking in G2. **(a)** Cell cycle distribution of asynchronous (AS) or synchronised cultures (as described in section 2.4.2) of B16 cells in specific stages of the cell cycle using flow cytometry. **(b)** The levels of phosphorylated Tbx2 increase during G2 in B16 cells. Protein extracts (62 µg) from either AS or synchronised cells, as described in (a), were analysed by 7.5% SDS-PAGE and western blotting using an anti-Tbx2 antibody to detect phosphorylated Tbx2. Levels of cyclin B1, cyclin A and total p38 were assessed by loading 20 µg of protein and analysis by 12% SDS-PAGE and western blotting with appropriate antibodies.

it may be phosphorylated by a kinase(s) that is active at this phase. Two possible candidates were cyclin A/Cdk2 and cyclin B1/Cdk1 which are both required for the events of G2 and M.

Since cyclin A and B1 are the regulatory subunits of these kinases the protein samples used in the above experiment were analysed by western blotting using antibodies to these cyclins. The results show that both cyclin A and B1 levels peak in G2. Interestingly, while the cyclin A levels are still high in M-phase the cyclin B1 levels drop to undetectable levels at this phase. The latter result was unexpected since cyclin B1 is regarded as the most important mitotic cyclin but was reproducible in many experiments using mouse B16 melanoma cells. To confirm the results obtained above, the experiment was repeated in B1Tbx2 cells, a mouse 3T3 fibroblast cell line expressing a low level of exogenous SV5-tagged Tbx2 (Prince et al., 2004). The conditions used to synchronise B16 cells in the different phases of the cell cycle were found to be effective for the B1Tbx2 cells (Fig. 3.11a). Results shown in Figure 3.11b reveal that changes in the levels of Tbx2, cyclin A and B1 are similar to those seen in B16 cells. These results suggest that both endogenous and exogenous Tbx2 are regulated similarly during the cell cycle and that the regulation of cyclin A and B1 were the same in these two mouse cells.

In the next set of experiments, the regulation of TBX2, cyclin A and B1 were investigated during the various phases of the cell cycle in human cells. Our laboratory has previously shown that while TBX2 is expressed in normal lung fibroblasts it is downregulated in several transformed human lung fibroblasts including a γ-radiated transformed WI-38 fibroblast cell line (CT-1). The CT-Tbx2 cell line was established by re-expressing TBX2 into the CT-1 cell line (Teng et al., 2007) and these cells were synchronised at specific phases of the cell cycle as previously described in section 3.2.1. Protein extracts from these cells were analysed by western blotting using the appropriate antibodies. Results are depicted in Figure 3.12. In these cells, the level of phosphorylated TBX2 was highest in G2 and hence matched the results obtained in the mouse B16 melanoma and B1Tbx2 fibroblast cells. However, the high level of phosphorylated TBX2 persisted in M-phase which contrasted with the results obtained for the mouse cells (see Figs. 3.10b and 3.11b). Interestingly, in these cells the level of the upper band was significantly more intense than the lower band, suggesting that the protein is almost completed phosphorylated in M-phase. These results suggested that either TBX2/Tbx2 protein levels are regulated differently in mouse or human cells or that ectopically expressed TBX2 in the CT-Tbx2 cells are regulated differently. To test the latter, the normal WI-38 lung fibroblast cell line that expresses high levels of TBX2 was included in these experiments. Successful synchronisation of these cells in mitosis was obtained and the results shown in Figure 3.12b indicate that, as seen in CT-Tbx2 cells, high levels of phosphorylated TBX2 are present at M-phase. As previously observed for the CT-Tbx2 cells the intensity of the upper band was also shown to be significantly higher in M-phase. These results suggested that TBX2/Tbx2 may be regulated differently in mouse and human cells, however, several mouse and human cell lines would have to be analysed in order to clarify this issue. Interestingly, when comparing the levels of cyclin A and B1 in the CT-Tbx2 cell line synchronised in the various phases of the cell cycle it is evident that they are regulated differently than those in the mouse cell lines examined in this study. This discrepancy could not be explained by a lack of specificity or sensitivity of the antibody to cyclin B1 used in this study. The result may however reflect differences in the

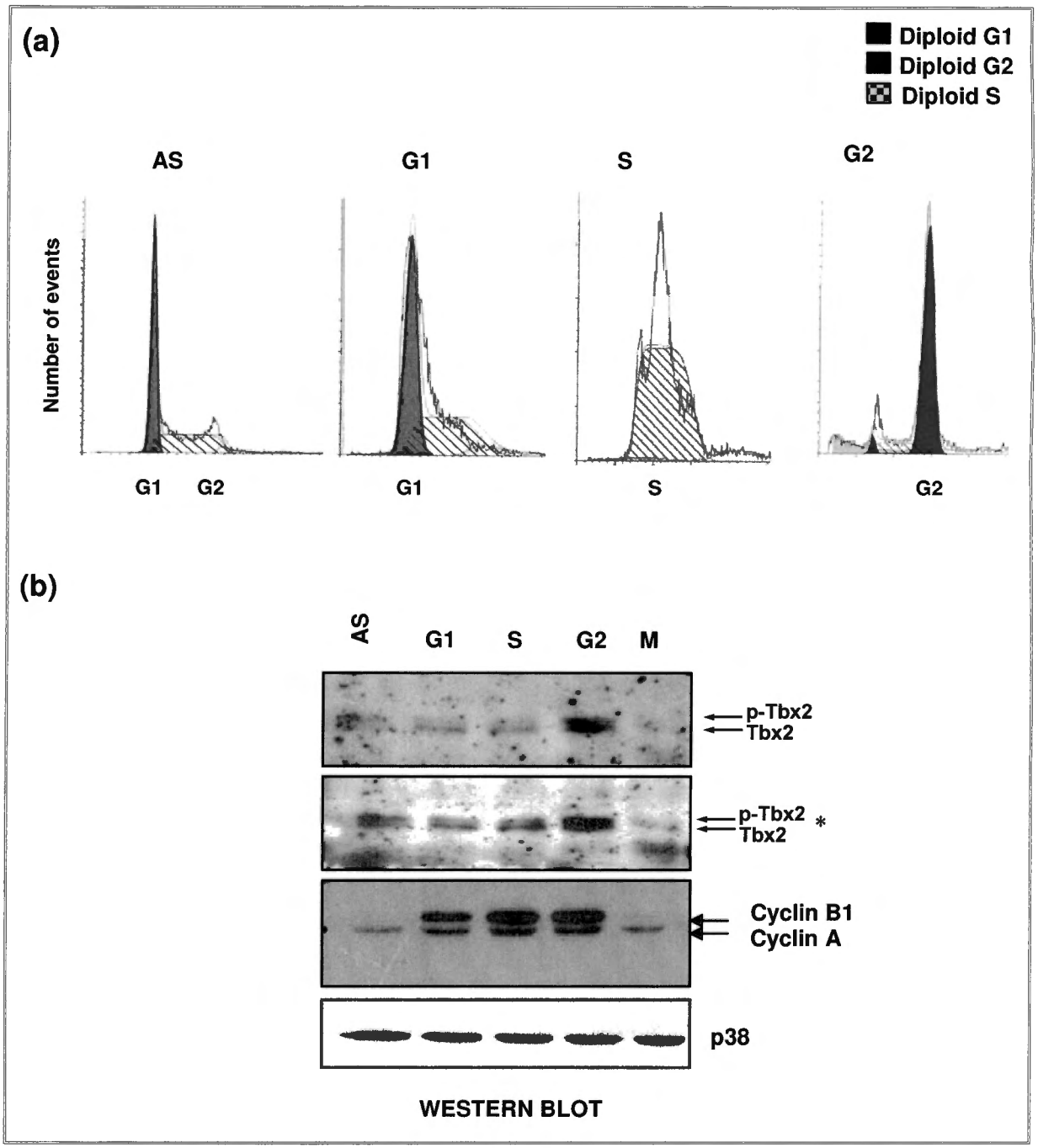


**Figure 3.11.** The levels of phosphorylated Tbx2 increases during G2 in the B1Tbx2 mouse fibroblast cell line. **(a)** Cell cycle distribution of asynchronous (AS) or synchronised cultures (as described in section 2.4.2) of cells in specific stages of the cell cycle using flow cytometry. **(b)** The levels of phosphorylated Tbx2 increase during G2. Protein extracts (80 µg) from either AS or synchronised cells, as described in (a), were analysed by 7.5% SDS-PAGE and western blotting using an anti-Tbx2 antibody to detect phosphorylated Tbx2. Levels of cyclin B1, cyclin A and total p38 were assessed by loading 50 µg of protein from these samples by 12% SDS-PAGE and western blotting with appropriate antibodies.

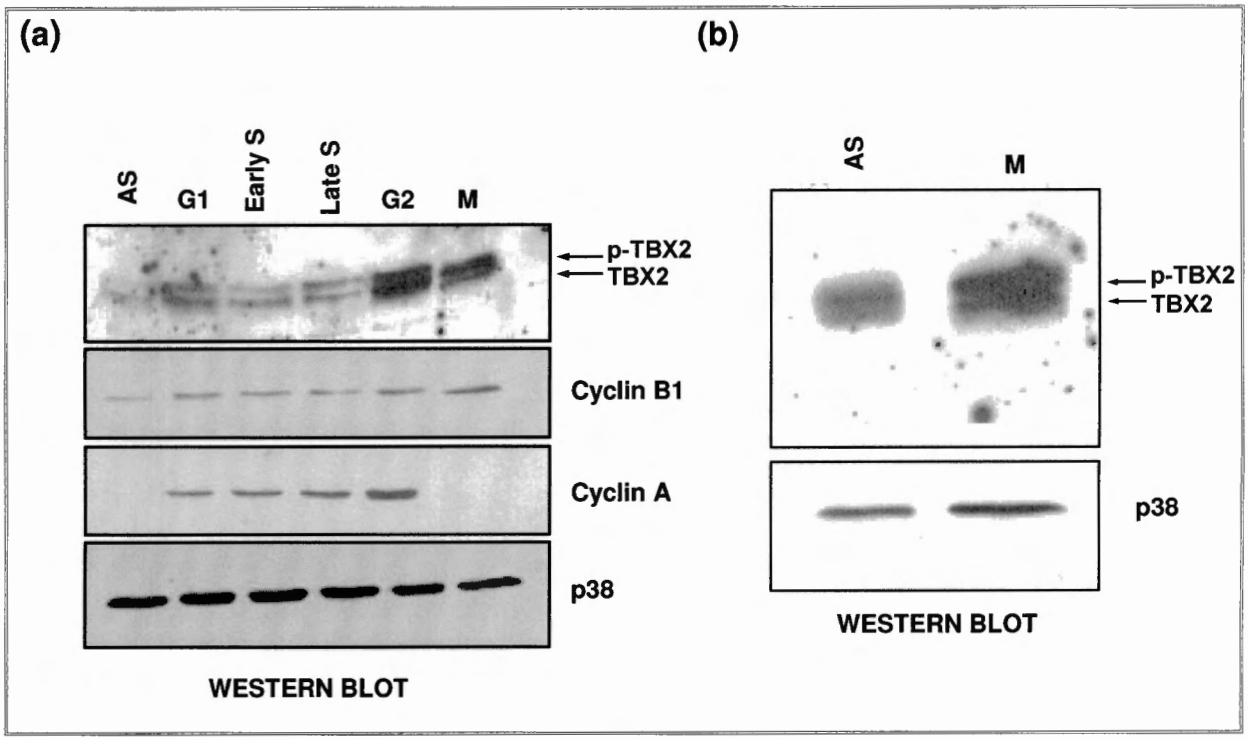


**Figure 3.12.** High levels of phosphorylated TBX2 persist in M-phase in human lung fibroblast cells. **(a)** The phosphorylation status of TBX2 in CT-Tbx2 cells is regulated in a cell cycle dependent manner. Protein extracts (40 µg) from both AS or synchronised CT-Tbx2 cells (see section 2.4.2) were analysed by 7.5% SDS-PAGE and western blotting to detect levels of phosphorylated TBX2. Early and late S-phase cells were obtained by a double thymidine block followed by release for 2 hr and 4hr, respectively. Levels of cyclin B1, cyclin A and total p38 were detected by loading 18 µg of protein from these samples by 12% SDS-PAGE and western blotting. **(b)** Phosphorylated TBX2 is present at high levels in normal WI-38 lung fibroblasts in M-phase. Protein extracts (27 µg) from AS and M-phase cells were analysed by SDS-PAGE and western blot analyses as described in (a).

regulation of cyclin B1 in mouse and human cells and may indicate that in mouse cells cyclin A can substitute for cyclin B1 in mitosis. This observation may be significant and while not further explored in this study is discussed in Chapter 4.

### 3.2.2 Endogenous TBX2/Tbx2 is phosphorylated by cyclin A/Cdk2 and cyclin B1/ Cdk1

Based on the above results it was hypothesised that TBX2/Tbx2 may be phosphorylated by cyclin A/Cdk2 and cyclin B1/Cdk1 which are the kinases active in G2 and M. This possibility was particularly attractive as the minimum phosphorylation sites for these kinases are ST/P and while there are no TP sites in the TBX2/Tbx2 protein, there are 13 SP motifs. To investigate whether Tbx2 is phosphorylated by cyclin A/Cdk2 and/or cyclin B1/Cdk1 in vivo, B16 cells were synchronised in S-phase as previously described in section 3.2.1 and released into G2 in the presence of olomoucine, a cyclin B1/Cdk1 and cyclin A/Cdk2 inhibitor (Veselý et al., 1994). Protein extracts from these cells were analysed by western blotting using the appropriate antibodies and results are shown in Figure 3.13a. As expected, Tbx2 was present at high levels in cells synchronised in G2 but in the presence of olomoucine, a significant decrease in the levels of the protein was observed.

The next objective was to determine whether TBX2 levels were regulated by cyclin A/CDK2 and/or cyclin B1/CDK1 during M-phase in the human CT-Tbx2 cells. To this end, cells were synchronised in G2 and released into M-phase in the presence of olomoucine. Figure 3.13b shows that in the presence of olomoucine, the levels of phosphorylated TBX2 were significantly reduced. These results confirmed that the levels of TBX2/Tbx2 during G2 and M are regulated by phosphorylation by cyclin A/Cdk2 and/or cyclin B1/Cdk1.

### 3.2.3 Subcellular localisation of TBX2/Tbx2 is regulated during the cell cycle

Since the subcellular localisation of TBX2 was shown to be regulated by p38 phosphorylation, it was possible that the subcellular localisation of TBX2/Tbx2 may also be regulated during the cell cycle. This possibility was investigated by synchronising the B16 melanoma and CT-Tbx2 cells in G1, S and G2 as described in 3.2.1 and examining the localisation of TBX2/Tbx2 by immunofluorescence. Figure 3.14 shows that Tbx2 was located in both the nucleus and cytoplasm in B16 melanoma cells synchronised in G1. In cells synchronised in S-phase, Tbx2 was observed primarily, although not exclusively, in the nucleus and by G2 the protein was almost exclusively nuclear.

Given the differences observed in the regulation of TBX2 in human cells in this study, the subcellular localisation of TBX2 was also investigated in CT-Tbx2 cells. Figure 3.15 shows that TBX2 is exclusively cytoplasmic in asynchronous and G1 arrested cells. Interestingly, when these cells were synchronised in S-phase the protein was either cytoplasmic or nuclear but never both. Importantly, almost all cells blocked in G2 exhibited only nuclear staining for TBX2. To confirm that the above results were not due to the chemicals used to synchronise the cells, immunofluorescence was performed on asynchronous CT-Tbx2 cells using anti-Tbx2 and co-stained with an antibody specific for Ser10-phosphorylated histone H3. Phosphorylation of H3

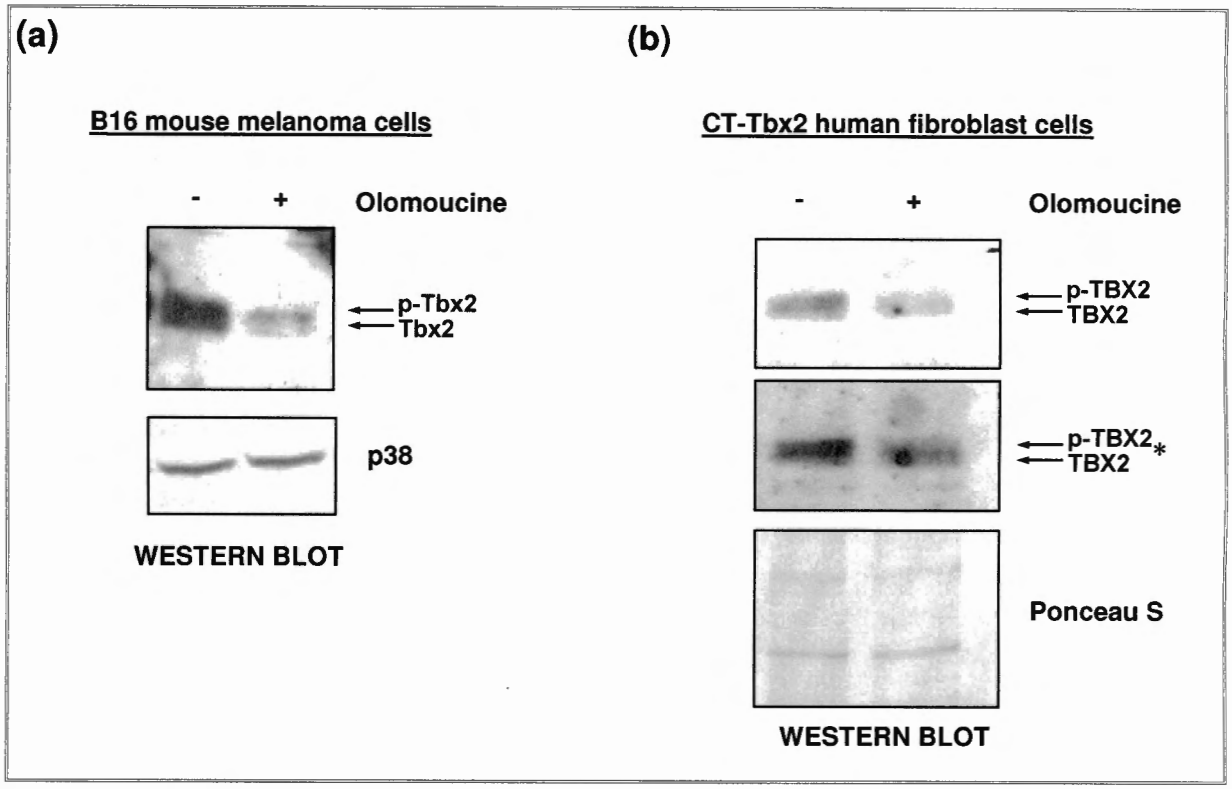


**Figure 3.13.** TBX2/Tbx2 is phosphorylated by the cyclin dependent kinases, cyclin A/Cdk2 and cyclin B1/Cdk1. **(a)** Tbx2 is phosphorylated in G2 by cyclin A/Cdk2 and/or cyclin B1/Cdk1. B16 cells synchronised in S phase as described in section 2.4.2 were released from the arrest in the presence of 80 µM olomoucine for 2 hr. Protein extracts (70 µg) were analysed on a 7.5% SDS-PAGE and subjected to western blotting to detect the phosphorylated form of Tbx2 using an anti-Tbx2 antibody. Levels of total p38 were assessed by loading 35 µg of protein from these samples by 12% SDS-PAGE and western blotting. **(b)** TBX2 is phosphorylated in vivo by cyclin A/CDK2 and/or cyclin B1/CDK1 during M-phase. CT-Tbx2 cells synchronised in M-phase were released from the arrest for 6 hr in the presence of 50 µM olomoucine. Protein extracts (26.5 µg) were analysed on a 7.5% SDS-PAGE as described in (a) above. As a loading control the membrane for the above western blot was stained with Ponceau S. (*) Indicates a longer exposure of the blot.

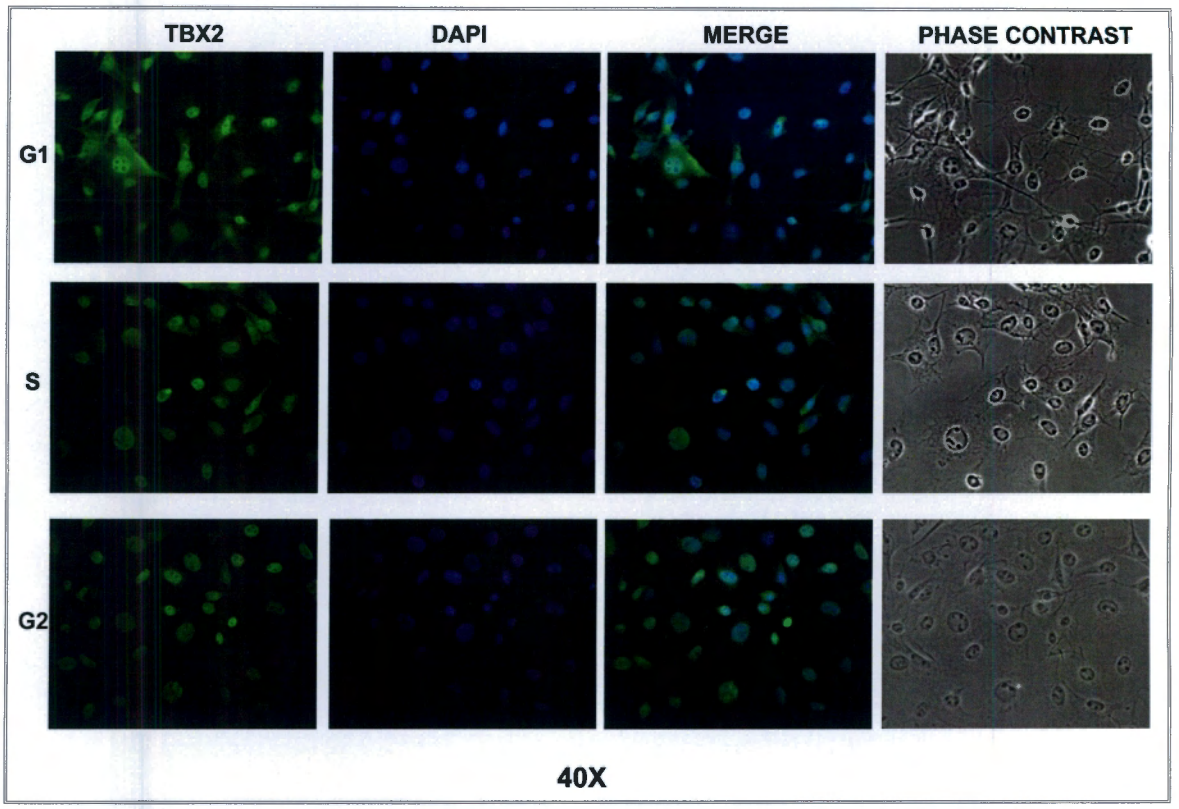


**Figure 3.14.** The subcellular localisation of Tbx2 is regulated during the cell cycle in mouse B16 cells. Cells synchronised in G1, S and G2 as described in section 2.4.2 were visualised by immunofluorescence at 40X magnification using an anti-Tbx2 antibody. All cells were co-stained with DAPI, to determine the location of the nuclei. Phase contrast images were photographed at the indicated magnification.

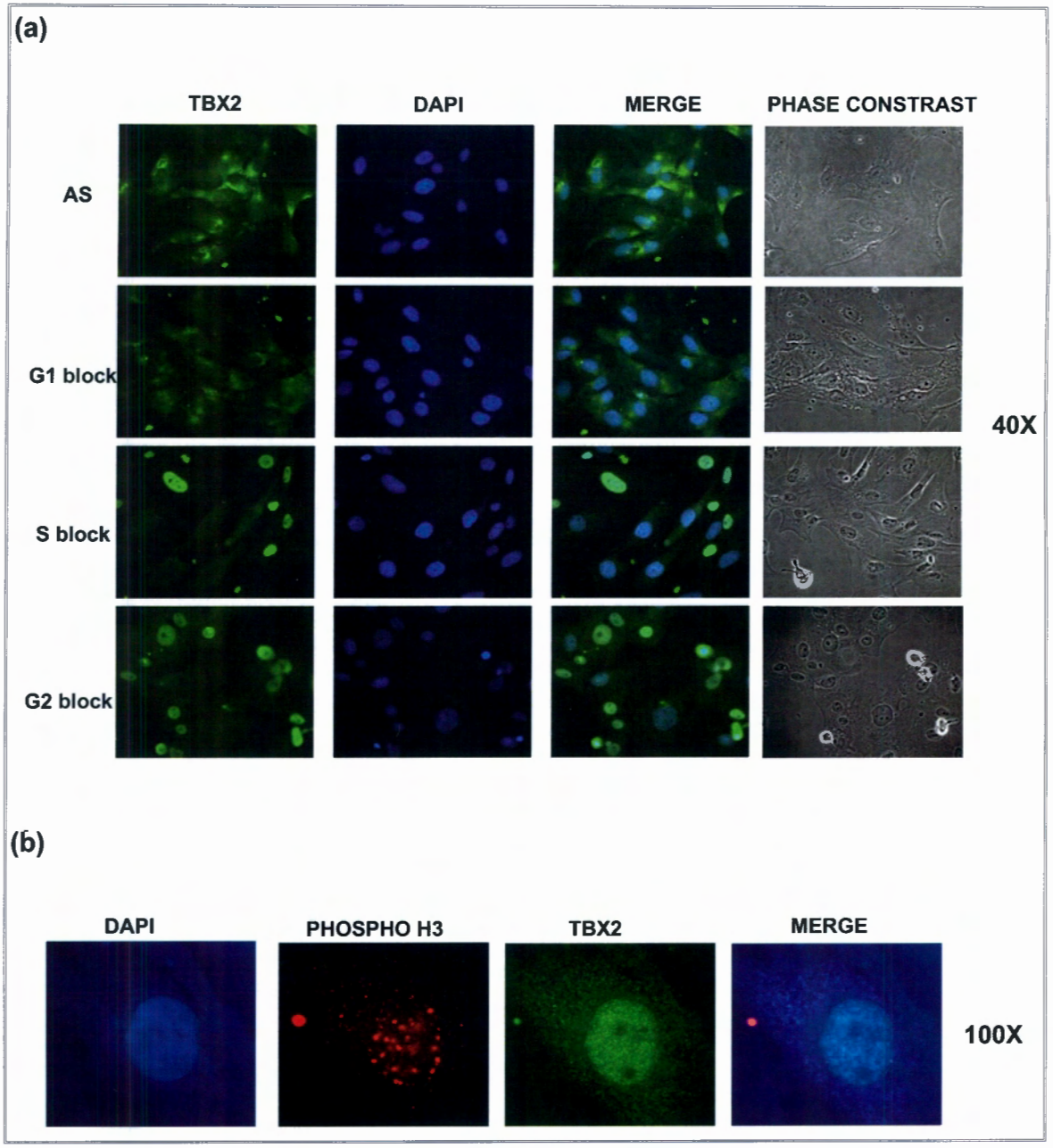


**Figure 3.15.** TBX2 localises to the nucleus in human fibroblast cells going through G2. **(a)** CT-Tbx2 cells were synchronised as described in Fig. 3.12a. Cells were visualised by immunofluorescence at 40X magnification using an antibody to Tbx2. Phase contrast images of the CT-Tbx2 were captured at the indicated magnification. **(b)** TBX2 co-localises with phospho-Histone 3 (H3) in the nuclei of G2 cells. Asynchronous CT-Tbx2 cells were stained with TBX2 and phospho-H3 which generates a specific punctate pattern of staining during G2. The fluorescent images depicted were captured at 100X magnification. The merged image is an overlay of the TBX2, DAPI and phospho-H3 stain. All cells were stained with DAPI, to determine the location of the nuclei.

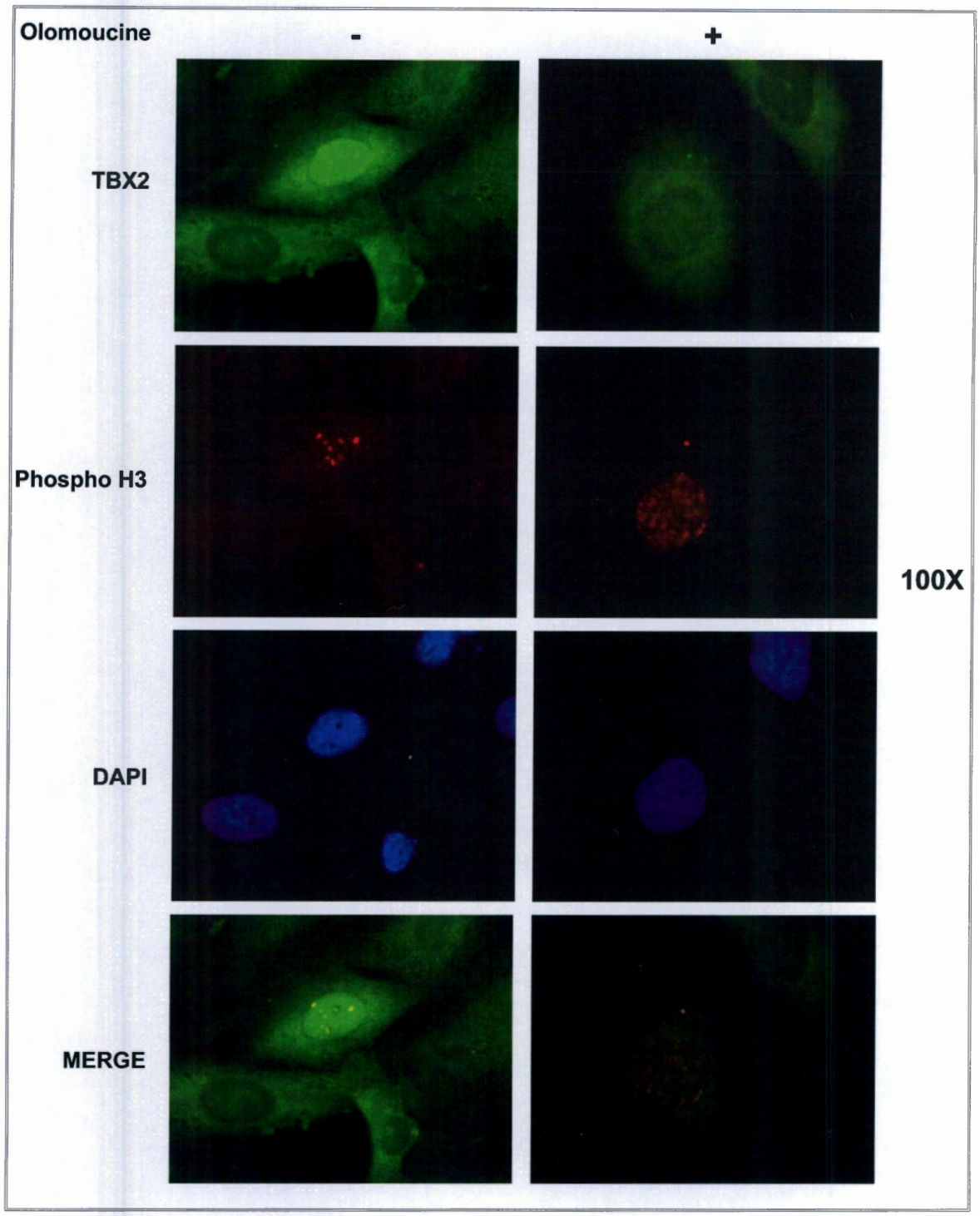


**Figure 3.16.** Phosphorylation by cyclin A/CDK2 and/or cyclin B1/CDK1 regulates the nuclear localisation of TBX2 at G2. Asynchronous CT-Tbx2 cells were treated for 4 hr with 50 μM olomoucine and analysed by immunofluorescence using anti-Tbx2 and Ser10-phosphorylated H3 antibodies. All cells were stained with DAPI. The panels represent images captured at 100X magnification. The merged image is an overlay of the TBX2 and phospho-H3 stain.

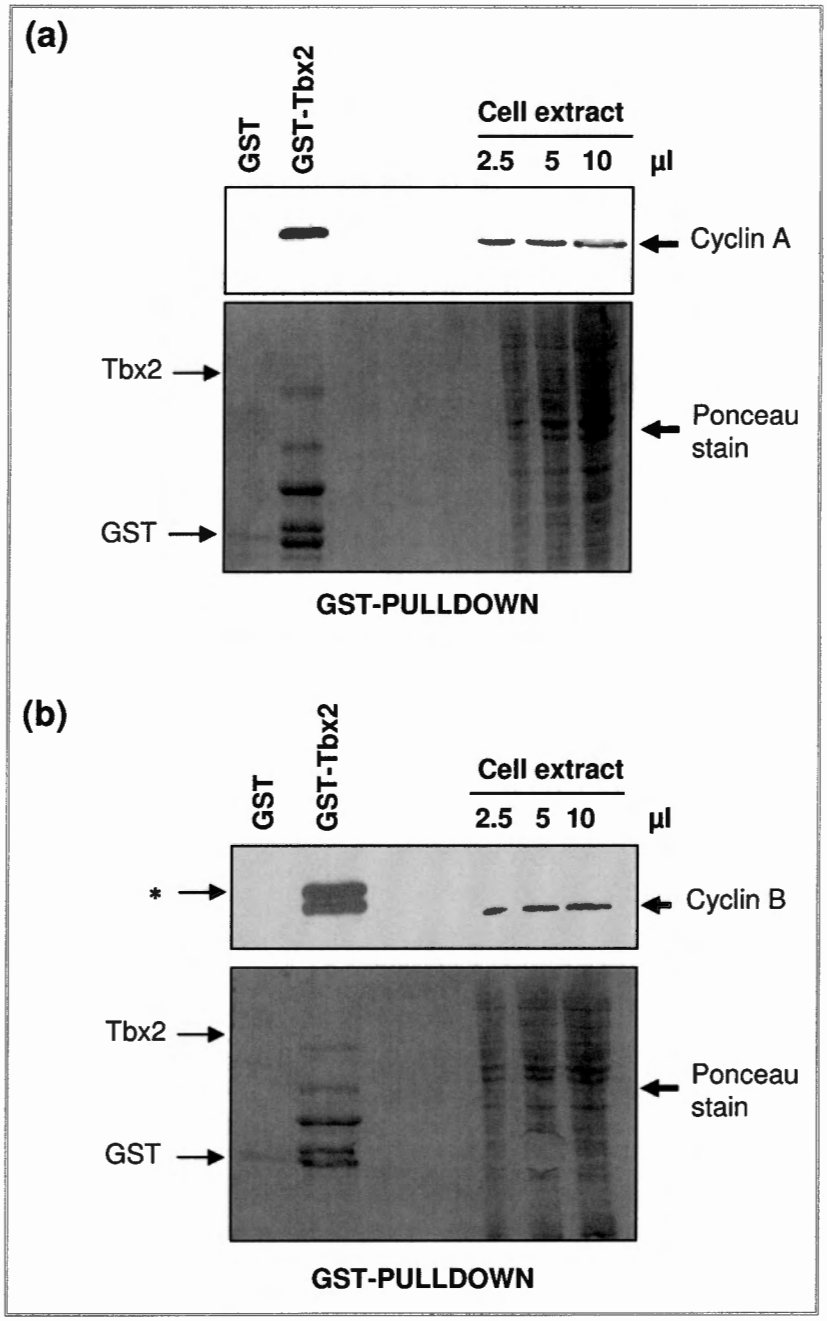


**Figure 3.17.** Cyclin A/Cdk2 and cyclin B1/Cdk1 directly bind Tbx2 in vivo. **(a)** Interaction of Tbx2 and cyclin A/Cdk2. B16 cell extracts synchronised in G2 and enriched with cyclin A were incubated with either GST-Tbx2 or GST only, which served as a negative control. Unbound cell extracts were included as a positive control. To show that an equal amount of protein was used in each lane the membrane was stained with Ponceau S (lower panel). Tbx2-bound proteins were resolved by 12% SDS-PAGE and binding to the cyclin was detected using an antibody specific to cyclin A. **(b)** Tbx2 binds cyclin B1/Cdk1. B16 cell extracts was prepared and analysed as described in (a). Binding to cyclin B1 was detected using the appropriate antibody. A non-specific band, which cross-reacts with the cyclin B1 antibody, is indicated with an asterisk (*). The position of Tbx2 and GST are indicated on the membranes.

To map the region of Tbx2 that interacts with cyclin A, a series of [$^{35}$S]methionine-labeled Tbx2 deletion proteins were generated using an in vitro transcription/translation system. The [$^{35}$S]methionine-labeled Tbx2 proteins were individually incubated with either bacterially produced GST-cyclin A or GST only, which served as a negative control. The input (20% of the reaction) and cyclin A-bound proteins were analysed by 12% SDS-PAGE. Protein interaction with the labelled Tbx2 proteins was visualised by autoradiography and the results are shown in Figure 3.18. As expected, cyclin A bound full length Tbx2, and while the N-terminal Tbx2 fragments (amino acids 1-202 and 114-376) were able to bind cyclin A, no binding was observed for the C-terminal fragment (amino acids 371-711). Interestingly, the minimum region that cyclin A bound within the N-terminal Tbx2 fragment was amino acids 147-295 which contains the DNA binding domain (T-box).

Previous work generated by Dr S. Prince in our laboratory has already established that cyclin B1 binds Tbx2 within its DNA binding domain and hence these experiments were not repeated. It would appear that both cyclin A and cyclin B1 bind Tbx2 within the same region, which interestingly does not contain any of the reported cyclin recognition motifs (KXL, RXL) and thus suggests a novel interaction between Tbx2 and the cyclins.

### 3.2.6 Tbx2 is phosphorylated by cyclin A/CDK2 and cyclin B1/CDK1 in vitro

Having established that cyclin A and cyclin B1 bind to Tbx2, the next objective was to determine whether Tbx2 was phosphorylated by the cyclin A/CDK2 and cyclin B1/CDK1 complexes. As mentioned earlier, SP motifs are the minimum potential target sites for phosphorylation by the CDKs and all 13 SP sites present in the Tbx2 protein had previously been mutated to alanine by site-directed mutagenesis. WT Tbx2 and the 13 mutated proteins were expressed as GST-tagged fusion proteins which were used as substrates for in vitro cyclin A/CDK2 kinase assays. These assays utilise [$\gamma$-$^{32}$P]ATP and thus the results were visualised by autoradiography. As in the p38 kinase assays, the GST-Tbx2 fusion proteins were expressed as either N-terminal (1-371) or C-terminal (371-711) proteins (Fig. 3.19a).

The results in Figure 3.19b (i) indicate that whereas the WT GST-Tbx2 N-terminal protein (1-371) was phosphorylated by cyclin A/CDK2, the C-terminal (371-711) protein was not. There are three SP motifs within the N-terminal protein that may be CDK2 target sites. However, whereas S342A had no effect on phosphorylation of the N-terminal portion of Tbx2, the S192A and S336A mutations both reduced phosphorylation by the cyclin A/CDK2 complex. To confirm that these two sites were cyclin A/CDK2 target sites, a S192A:S336A double mutant was generated, expressed as a GST-tagged fusion protein and included as a substrate together with the proteins tested above in a kinase assay. Figure 3.19b (ii) shows that whereas the single mutations S192A and S336A reduced phosphorylation of the N-terminus of Tbx2, when they were both mutated phosphorylation was severely reduced to almost background levels. GST alone was used as a negative control. The lower panels in Figure 3.19b show the gels stained with Coomassie Blue indicating that equivalent amounts of GST-fusion proteins were used in these experiments. The bar graphs, which depict densitometric values of radioactive

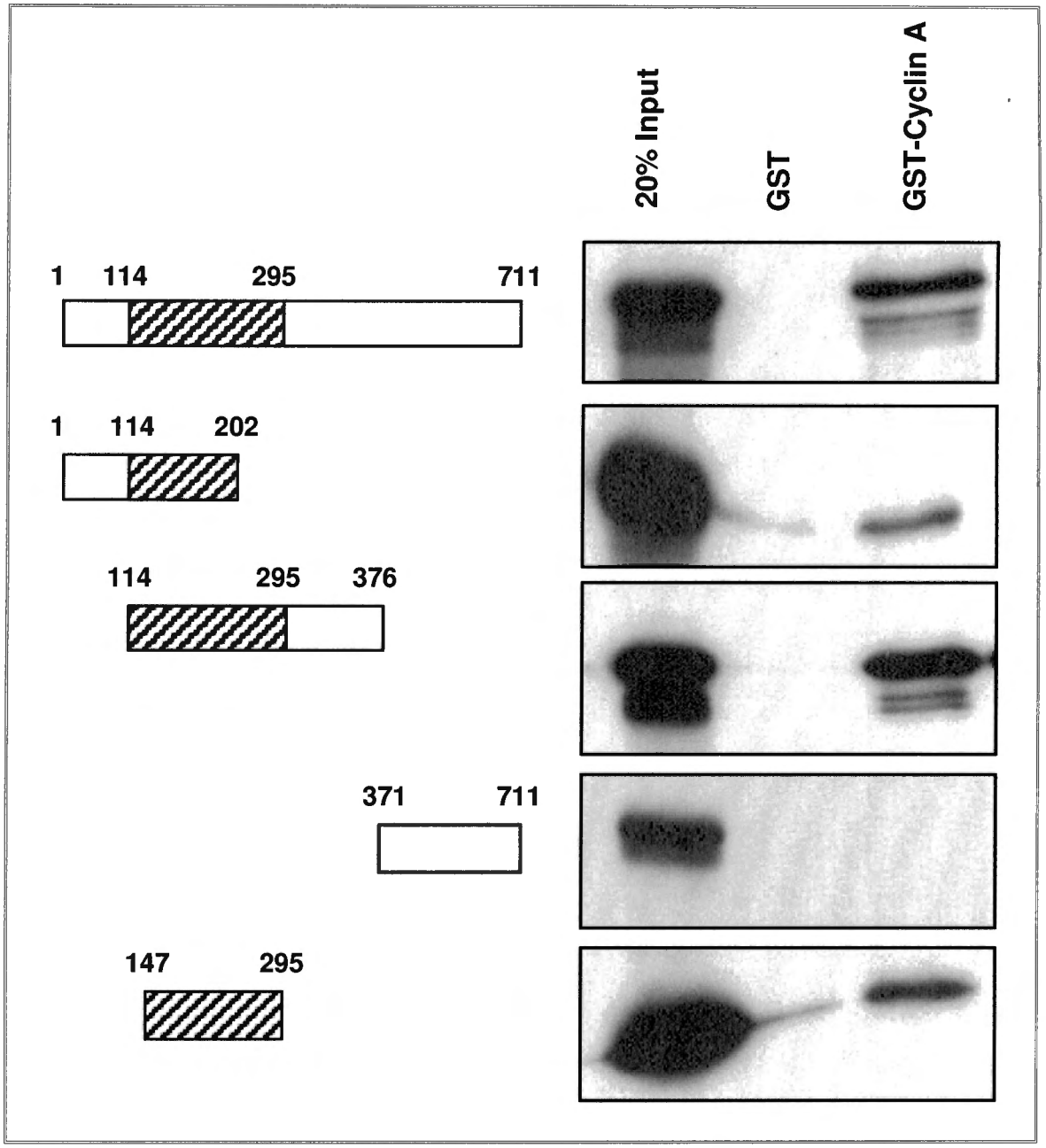


**Figure 3.18.** Cyclin A binds Tbx2 within the T-box. Schematic diagram of the [$^{35}$S]methionine-labeled WT and Tbx2 deletion proteins generated in vitro using the TNT quick coupled transcription-translation system (left panel, not to scale). The indicated [$^{35}$S]methionine-labeled Tbx2 deletion proteins were used to map the minimal interaction domain that cyclin A requires to bind Tbx2 (right panel). 20% of the reaction was retained as input and the remainder of the reaction was incubated with an equal amount of GST or GST-Cyclin A. Tbx2 in both the input and GST-containing lanes were detected by autoradiography. The striped box indicates the DNA-binding domain of Tbx2.

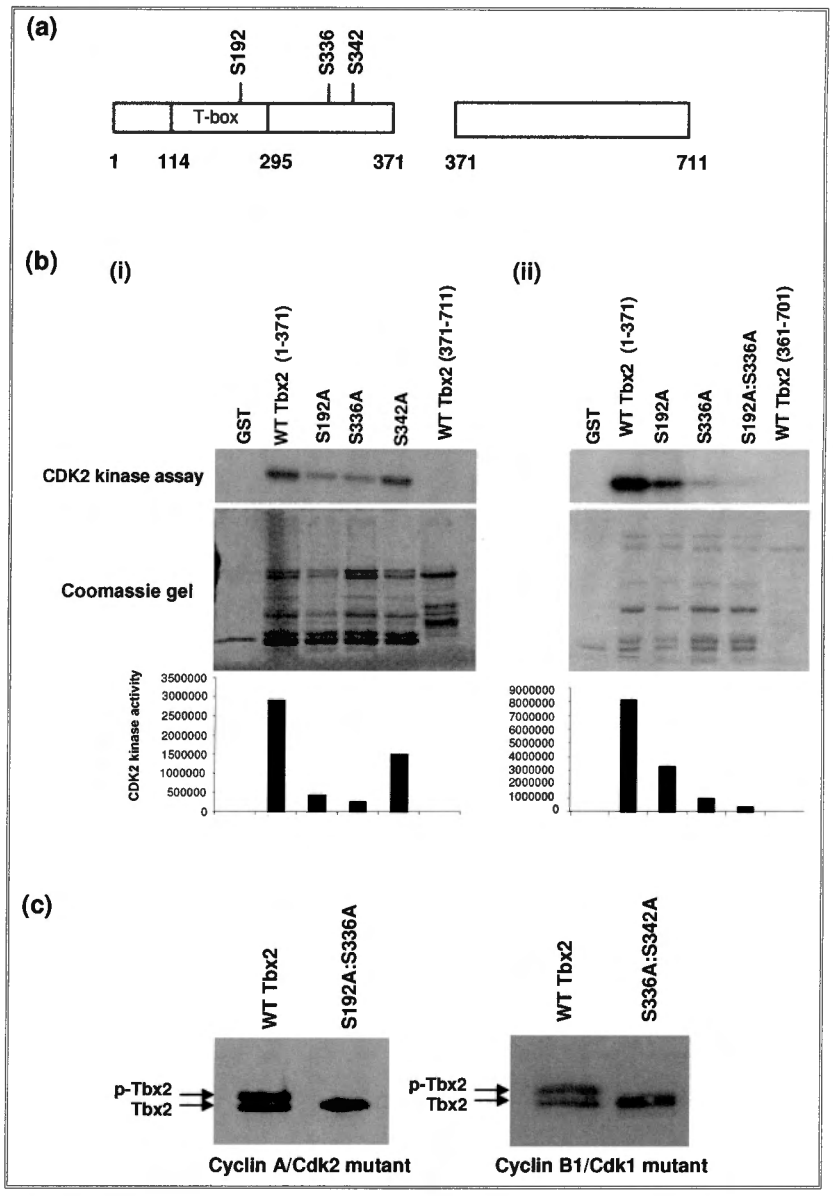


**Figure 3.19.** Tbx2 is phosphorylated by cyclin A/Cdk2 and cyclin B1/Cdk1 at specific serine residues in vitro and in vivo. **(a)** Schematic representation of the N-terminal (1-371) and C-terminal (371-711) Tbx2 proteins used as substrates in CDK2 kinase assays. **(b)** Mapping the Cyclin A/CDK2 kinase target sites within Tbx2. In vitro kinase assays were performed using purified GST-Tbx2 fusion proteins as substrates in the presence of the recombinant activated cyclin A/CDK2 kinase and [$\gamma$-$^{32}$P]ATP. Kinase assays using the indicated Tbx2 proteins are shown in the upper panel after 12% SDS-PAGE and autoradiography. The lower panels show the same gels stained with Coomassie Blue indicating that equivalent amounts of protein were used in each assay. The bar graphs shows densitometric values of radioactive levels measured for each construct in the kinase assay. **(c)** Mutating the cyclin A/Cdk2 and cyclin B1/Cdk1 kinase sites affects the phosphorylation of Tbx2 in vivo. SV5 epitope-tagged Tbx2 or the cyclin A/Cdk2 (S192A:S336A) and the cyclin B1/Cdk1 (S336A:S342A) mutant were transiently transfected in COS-7 cells and an equal amount of protein from each sample was separated by 7.5% SDS-PAGE and western blotting to analyse the phosphorylation status of Tbx2 using an anti-SV5 antibody. p–Tbx2 is phosphorylated Tbx2.

levels measured for each substrate, confirmed the visual results of the kinase assays. These results show that Tbx2 is phosphorylated specifically by the cyclin A/CDK2 kinase at S192 and S336 in vitro.

Previous work done by Dr S. Prince using the same substrates as above in an in vitro kinase assay with cyclin B1/CDK1 showed that Tbx2 is phosphorylated by this kinase complex at serine residues 336 and 342. Taken together, the results show while cyclin A/CDK2 and cyclin B1/CDK1 target a common site (S336) within the Tbx2 protein, they each have an additional distinct target site.

### 3.2.7 Tbx2 is phosphorylated at the cyclin A/Cdk2 and cyclin B1/Cdk2 target sites in vivo

To determine whether the cyclin A/Cdk2 and cyclin B1/Cdk1 target sites are sites for phosphorylation in vivo, Tbx2 mutants were generated in which the cyclin A/Cdk2 or cyclin B1/Cdk1 target sites were mutated to alanine within the full-length Tbx2 protein (Tbx2 S192A:S336A and Tbx2 S336A:S342A, respectively). COS-7 cells were transfected with vectors expressing either SV5-tagged WT Tbx2 or SV5-tagged mutant proteins and the phosphorylation status of Tbx2 analysed by western blotting. Figure 3.19c shows that unlike the two bands seen for WT Tbx2, both the cyclin A/Cdk2 (S192A:S336A) and cyclin B1/Cdk1 (S336A:S342A) mutant proteins showed up as a single band. These results suggest that the identified cyclin A/Cdk2 and cyclin B1/Cdk1 target sites are indeed phosphorylated in vivo.

### 3.2.8 Functional significance of Tbx2 phosphorylation by cyclin A/Cdk2 and cyclin B1/Cdk1

To determine the functional consequences of Tbx2 phosphorylation by the cyclin A/Cdk2 and cyclin B1/Cdk1 kinases, the next objective was to investigate the effect of phosphorylation by these kinases on Tbx2 (a) protein stability and (b) ability to repress transcription.

#### 3.2.8.1 Phosphorylation by cyclin B1/Cdk1 but not cyclin A/Cdk2 increases Tbx2 protein stability

To investigate the effect of cyclin A/Cdk2 and/or cyclin B1/Cdk1 phosphorylation on the stability of the Tbx2 protein, NIH 3T3 cells were transiently transfected with either WT Tbx2 or Tbx2 mutant proteins in which cyclin A/Cdk2 (S192:S336) or cyclin B1/Cdk1 (S336:S342) target sites were mutated to either alanine (A), to abolish phosphorylation or to glutamic acid (E), to mimic phosphorylation. The transfected cells were treated with cycloheximide over a period of 6 hr at the indicated times and protein analysed by western blot analyses using a SV5 antibody (Fig. 3.20). In order to accurately quantify total levels of Tbx2 protein, in this experiment cell lysates were separated by a 10% SDS-PAGE and hence Tbx2 is detected as a single band. The results obtained for WT Tbx2 and the cyclin A/Cdk2 mutants, (S192A:S336A and S192E:S336E), are shown in Figure 3.20a. Whereas the levels of the WT Tbx2 protein remained almost unchanged over the period of cycloheximide treatment, the levels of the Tbx2 S192A:S336A mutant protein decreased after 1 hr and continued to decline with increasing duration of cycloheximide treatment. These results suggest that abolishing phosphorylation at S192 and S336 affects the stability of the protein. However, levels of the pseudo-phosphorylated Tbx2

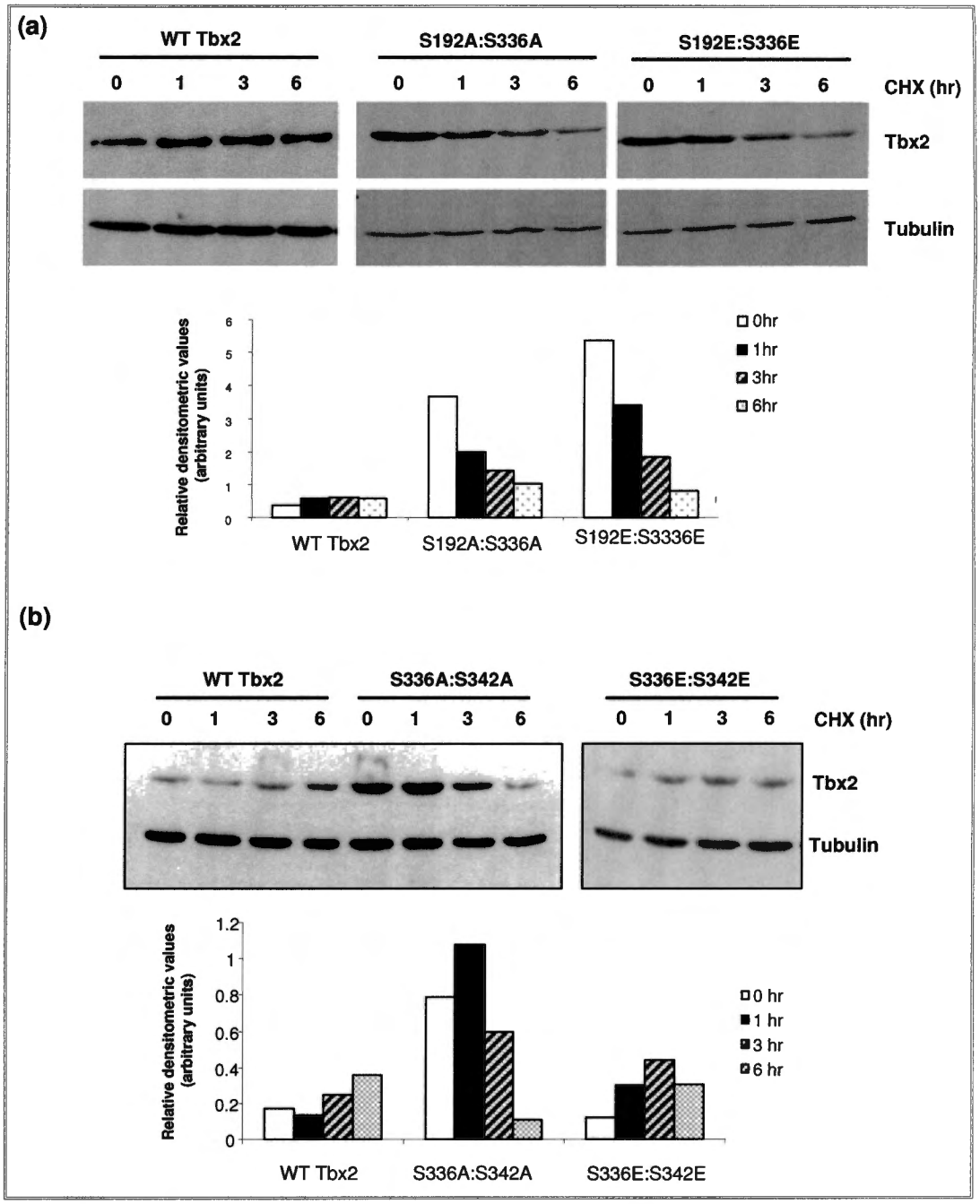


**Figure 3.20.** Pseudo-phosphorylation at the cyclin B1/Cdk1 but not cyclin A/Cdk2 target sites increases Tbx2 protein stability. **(a)** NIH 3T3 cells, transiently transfected with vectors expressing SV5 epitope-tagged WT Tbx2 and the cyclin A/Cdk2 Tbx2 mutants were incubated 48 hr post-transfection with 30 µg/ml CHX for the indicated times. To accurately detect total levels of the Tbx2 protein as a single band, an equal amount of protein from each sample was analysed by 10% SDS-PAGE and western blotting with anti-SV5 antibodies. The bar graph compares the intensity of the Tbx2 protein normalised to the loading control. **(b)** Western blot analyses of protein from NIH 3T3 cells transiently transfected with vectors expressing SV5 epitope-tagged WT Tbx2, the Tbx2 S336A:S342A mutant or the Tbx2 S336E:S342E mutant, and treated with 30 µg/ml CHX as described above. Proteins were analysed as described in (a) above.

S192E:S336E mutant protein behaved exactly the same as the Tbx2 S192A:S336A mutant protein, which was puzzling and a possible explanation for this is provided at the end of this section. The densitometric graphs in the lower panels compare the intensity of the Tbx2 protein normalised to the loading control.

Figure 3.20b shows results obtained for WT Tbx2 and the cyclin B1/Cdk1 mutants, (S336A: S342A) and (S336E:S342E). As seen in previous experiments, the levels of WT Tbx2 remained reasonably stable during the period of cycloheximide treatment and a similar result was obtained for the pseudo-phosphorylated Tbx2 mutant (S336E:S342E). In contrast, the Tbx2 S336A:S342A mutant exhibited significantly reduced protein levels after 3 hours and very low levels at 6 hours of cycloheximide treatment. These results indicate that phosphorylation of Tbx2 by cyclin B1/Cdk1 at serine residues 336 and 342 enhances Tbx2 protein stability.

It is important to point out at this stage that this study shows that serine 336 in Tbx2 is a target for the p38 kinase, cyclin A/Cdk2 and cyclin B1/Cdk1. Since the Tbx2 mutants that mimic phosphorylation by p38 and cyclin B1/Cdk1 are more stable than their A mutant counterparts, the question arises as to whether the effect seen for the mutant that mimics phosphorylation by cyclin A/Cdk2 is a consequence of mutating S192 located in the DNA binding domain. It is possible that substitution of serine residues with glutamic acid does not mimic exactly the effect of phosphorylation at these sites and that even a small difference between glutamic acid and a phosphate group(s) may have deleterious effects on a protein depending on the position of the substitution, with S192 being such an example.

### 3.2.8.2 Phosphorylation by cyclin B1/Cdk1 enhances the ability of Tbx2 to repress the p21 promoter

Tbx2 may be playing a critical role in the regulation of the cell cycle, particularly at the G2 and/or M phase. One possible mechanism of how Tbx2 may contribute to the maintenance of cell cycle progression is postulated to be through its ability to repress transcription of the cyclin-dependent kinase inhibitor, *p21*. As previously discussed (see Chapter 1), p21 is critical for mediating a cell cycle arrest through its association with appropriate cyclin/Cdk complexes in response to cellular stress. It was therefore hypothesised, given the importance of p21 in the regulation of key cellular processes such as the cell cycle, senescence and cancer that a key experiment would be to test the ability of the cyclin A/Cdk2 and cyclin B1/Cdk1 Tbx2 mutants to repress the transcriptional activity of *p21*. To this end, COS-7 cells were co-transfected with either WT Tbx2 or the Tbx2 mutants in which the cyclin A/Cdk2 and cyclin B1/Cdk1 target sites had been mutated to alanine (A) or to glutamic acid (E) together with a *p21* promoter-luciferase reporter. The results, in which each construct was tested in duplicate, are shown in Figure 3.21a and 3.21b.

Figure 3.21a depicts the result of three different experiments, which shows the effect of the cyclin A/Cdk2 mutants on the *p21* promoter. While co-transfection with a WT Tbx2 expression vector consistently resulted in an approximately 3 fold repression of the *p21* promoter, the effect of the cyclin A/Cdk2 mutants was however not reproducible. Both the Tbx2 (S192A:

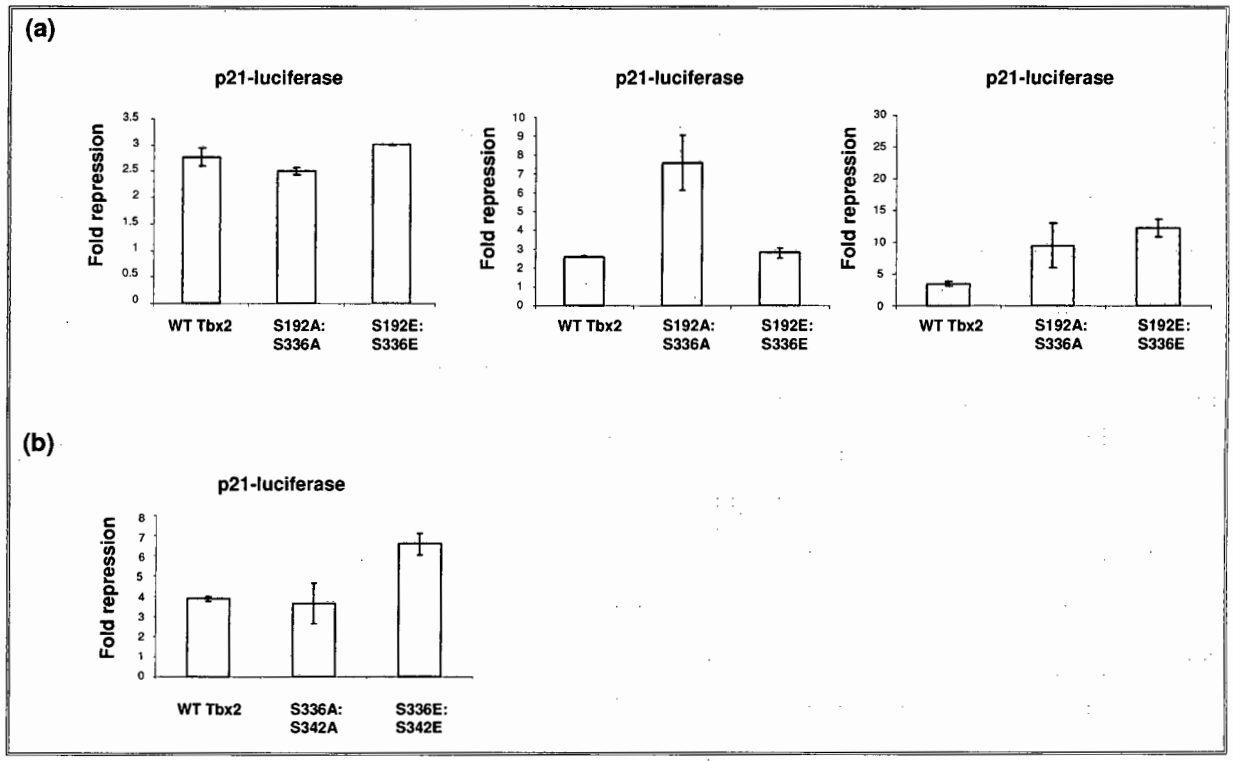


**Figure 3.21.** Pseudo-phosphorylation at the cyclin B1/Cdk1 target sites enhances the ability of Tbx2 to repress *p21*. The *p21* promoter-luciferase reporter (700 ng) was co-transfected into COS-7 cells either with the empty pCMV (200 ng) vector or with the **(a)** Tbx2 expression vectors in which the cyclin A/Cdk2 sites had either been abolished (S192A:S336A) or pseudo-phosphorylated (S192E:S336E) and **(b)** the Tbx2 expression vectors in which the cyclin B1/Cdk1 sites had either been abolished (S336A:S342A) or pseudo-phosphorylated (S336E:S342E) and luciferase acitivity determined. Error bars represent means ± standard deviation.

S336A) and (S192E:S336E) mutants was shown to either behave similarly to WT Tbx2 or exhibit increased repression on the *p21* promoter. As previously discussed in section 3.2.8.1, a possible explanation for these inconsistent results could be that substitution of S192 located in the DNA binding domain of Tbx2 with either A or E may disrupt the structure of the Tbx2 protein. The results from these experiments are thus inconclusive.

In contrast, in these experiments the Tbx2 (S336E:S342E) mutant that mimics phosphorylation by cyclin B1/Cdk1 consistently exhibited increased repression on the *p21* promoter (Fig. 3.21b). These results were also shown to be reproducible using CAT reporter assays (data not shown). The results suggest that phosphorylation of Tbx2 by cyclin B1/Cdk1 kinase enhances its transcriptional repression on the *p21* promoter.

# CHAPTER 4: DISCUSSION

Members of the T-box family play important roles in a wide variety of developmental processes and two family members, TBX2 and TBX3, have been shown to contribute to the growth and survival of several cancers. Importantly, TBX2 and TBX3 were identified as immortalising genes capable of bypassing replicative senescence through their ability to repress *p19$^{ARF}$* and *p21* (Jacobs et al., 2000; Brummelkamp et al., 2002; Lingbeek et al., 2002; Prince et al., 2004). Despite their important roles in a number of biological processes, very little is known about the biochemical pathways that regulate the levels and transcriptional activity of T-box factors. In view of the detrimental consequences resulting from altered levels of T-box proteins, as seen in both developmental disorders and in certain cancers, the need to identify such pathways is important. This study has therefore focused on identifying signal transduction pathways and kinases that regulate the levels and activity of TBX2. To this end, it was firstly important to establish whether TBX2 is phosphorylated in vivo. Protein extracts from the MCF-7 breast cancer cell line were therefore treated with and without shrimp alkaline phosphatase and results from western blot analyses showed that the endogenous TBX2 protein is expressed as two bands, with the top band representing the phosphorylated protein. Furthermore, the TBX2 protein sequence was shown to have 13 SP motifs that are potential target sites for several kinases and this study shows that some of them are phosphorylated by the stress-induced p38 kinase, and the G2/M cyclin A/Cdk2 and cyclin B1/Cdk1.

## TBX2 is phosphorylated by the p38 MAP kinase in response to stress induced by UVC irradiation

Cellular senescence can be triggered by alterations of telomeres as well as in response to a variety of stresses, such as DNA damage caused by irradiation and cytotoxic drugs (reviewed in Roninson, 2003). The p38 signalling pathway plays an important role in diverse cellular responses such as cell cycle progression, differentiation, apoptosis (reviewed in Zarubin and Han, 2005) and has also been identified as a senescence signalling pathway which is activated in response to both telomere dependent and independent senescence (Iwasa et al., 2003; Haq et al., 2002; Wang et al., 2002). In response to DNA-damaging agents, such as UV irradiation, the p38 MAP kinase signal transduction cascade is initiated to inhibit cell cycle progression and to activate DNA-repair processes, thereby maintaining the integrity of the genome. The present study provides several lines of evidence that TBX2 is phosphorylated by the p38 MAP kinase in response to stress induced by UVC irradiation.

Firstly, phosphorylation of TBX2 was shown to increase with increasing doses of UVC irradiation and this was reversed in the presence of the p38 inhibitor, SB203580. Secondly, using in vitro kinase assays, Tbx2 was shown to be phosphorylated at serine residues 336, 623 and 675 and these sites were confirmed to be phosphorylated in vivo. Furthermore, in response to UVC treatment the phosphorylation status of the Tbx2 mutant (S336A:S623A:S675A) remained unchanged, confirming that the sites identified were the only sites targeted for phosphorylation by p38.

Importantly, phosphorylation by the p38 MAP kinase was shown to have a significant effect on TBX2 function. For example, the Tbx2 protein appears less stable when the three p38 serine target sites were mutated to block phosphorylation. This effect of p38 phosphorylation on the stability of its substrates has previously been reported for the G1 proteins, p53 and cyclin D1. Whereas p38 phosphorylation of p53 led to increased protein stability, phosphorylation of cyclin D1 by this kinase resulted in ubiquitin-dependent degradation and consequently a G1 arrest (Casanovas et al., 2000; Bulavin et al., 1999; Keller et al., 1999). To confirm that phosphorylation by p38 at serine residues 336, 623 and 675 protects Tbx2 from degradation it would be important to examine the stability of endogenous TBX2 in response to UV in the presence of SB203580 using pulse-chase labeling.

Moreover, *TBX2* mRNA levels also increased in response to UVC irradiation but the mechanism(s) for this still needs to be elucidated. It is tempting to speculate that the ubiquitous bHLH-LZ transcription factor USF-1 may be involved. This is possible because both TBX2 and USF-1 are expressed in melanocytes and USF-1 has been shown to play an important role in mediating the tanning response, which protects skin from UV-induced DNA damage (Carreira et al., 1998; Galibert et al., 2001). During the tanning response, phosphorylation of USF-1 by the p38 MAP kinase results in an increase in USF-1 binding to conserved E and M box motifs and consequently activation of genes that encode enzymes involved in the melanin synthesis pathway (Galibert et al., 2001). The *Tbx2* promoter has been shown to possess an E box (Carreira et al., 2000) and it would be interesting to see if it is capable of responding to USF-1, particularly in response to UV irradiation. To test this theory, future work should determine whether USF-1 is able to bind the *TBX2* promoter in vitro and in vivo using electromobility shift and chromatin immunoprecipitation assays respectively. In addition, the effect of USF-1 on the promoter activity of *TBX2* could be assessed by co-transfecting cells with a USF-1 expression construct and the TBX2-LUC reporter construct, UV irradiating the cells, and then measuring luciferase activity. It is anticipated that in response to UV irradiation the stress-responsive USF-1 transcription factor will bind and transciptionally activate the expression of *TBX2*.

TBX2 was shown to localise to both the cytoplasm and nucleus but upon UVC irradiation, phosphorylation of TBX2 by the p38 MAP kinase led to the predominant nuclear localisation of the protein. This result would suggest that the activity of TBX2 is controlled, in part, by the regulation of its subcellular localisation. This is not the first study to suggest the importance of the subcellular distribution of T-box factors in regulating their functional activity. For example, in *C. elegans*, tbx2 is involved in olfactory adaptation and the protein is mostly localised to the cytoplasm of olfactory and many pharyngeal neurons, suggesting that nuclear translocation may regulate its activity (Miyahara et al., 2004). Certain T-box family members have also been found to possess nuclear localisation signals (NLS), which may explain the shuttling of these proteins into the nucleus. In patients suffering from DiGeorge syndrome, the deletion of the NLS in Tbx1 has been shown to prevent the mutant protein from localising to the nucleus, resulting in haploinsufficiency (Stoller and Epstein, 2005). Furthermore, deletion of the Tbx3 NLS, RREKRK, at amino acids 292-297 results in mislocalisation of the protein to either perinuclear or cytoplasmic sites (Carlson et al., 2001).

Given the high degree of homology between TBX2 and TBX3, the human and mouse TBX2 protein sequences were screened for the TBX3 NLS. Not surprisingly, the TBX3 NLS was found to be conserved at exactly the same position in the TBX2 protein. It will therefore be interesting to investigate whether the identified NLS plays a role in shuttling TBX2 into the nucleus and whether phosphorylation of the protein by the p38 MAP kinase can regulate this process. A key experiment would thus be to delete the identified TBX2 NLS and in the presence and absence of UVC irradiation monitor the translocation of TBX2 to the nucleus using fluorescent microscopy. There is, however, another possible mechanism by which phosphorylation by p38-mediated phosphorylation can regulate the translocation of TBX2 into the nucleus. It is well established that the p38 kinase does not possess a NLS or nuclear export signal (NES) and that the shuttling of p38 to the nucleus occurs via specific docking interactions with NES- and NLS containing proteins, such as p38 activators, substrates or scaffold proteins (reviewed in Cyert, 2001). Therefore in response to UVC irradiation, it is also likely that active p38, in complex with NLS containing proteins, binds and phosphorylates TBX2 in the cytoplasm and enables its translocation to the nucleus as part of this complex.

The effect of UVC-induced p38 phosphorylation on TBX2 protein levels and nuclear localisation would suggest a role for this transcription factor in the stress signalling pathway. There has been a lot of controversy in the literature regarding the role and regulation of the Tbx2 target, *p21*, in response to UV-induced DNA damage. While certain studies suggest that p21 plays a positive role in the DNA damage repair pathway, other studies have suggested that its degradation is required for this process. The present study shows that in response to UVC irradiation, p21 protein levels are rapidly increased but decrease equally rapidly post-UVC treatment. This is consistent with recent reports that in response to DNA damage, p21 levels initially increase in order to establish a senescence-like growth arrest by binding to appropriate cyclin/Cdk complexes and by inhibiting the activity of proliferating cell nuclear antigen (PCNA) (Fig. 4.1a). Subsequently however, p21 has been shown to be downregulated which is thought to be required for the activation of PCNA which is a key component of the nucleotide excision repair (NER) pathway (Pan et al., 1995; Cooper et al., 1999; Bendjennat et al., 2003). One mechanism by which p21 is downregulated in response to UV-induced DNA damage has been shown to involve ubiquitin-dependent proteolysis (Bendjennat et al., 2003). The present study however shows that *p21* mRNA levels also decrease immediately in response to UVC irradiation with levels remaining low for up to 60 min post-treatment. This finding is consistent with a study by Wang et al (1999) which demonstrated that both p21 protein and mRNA levels are downregulated post-UV irradiation of MCF-7 cells. The observed decrease in *p21* mRNA levels post-UVC treatment correlated with an increase in TBX2 protein levels, which raised the possibility that TBX2 may be required to inhibit *p21* in the UV-stress signalling pathway. Indeed, in response to UVC irradiation TBX2 was shown to be a much stronger repressor of the *p21* promoter and mutating the three p38 serine target sites within TBX2 abrogated its ability to repress *p21*. These results suggest that TBX2 may be required to inhibit *p21* mRNA levels in the UV-induced DNA damage pathway which contributes to the downregulation of p21 protein levels as depicted in Figure 4.1. To test this model, a key experiment would be to determine

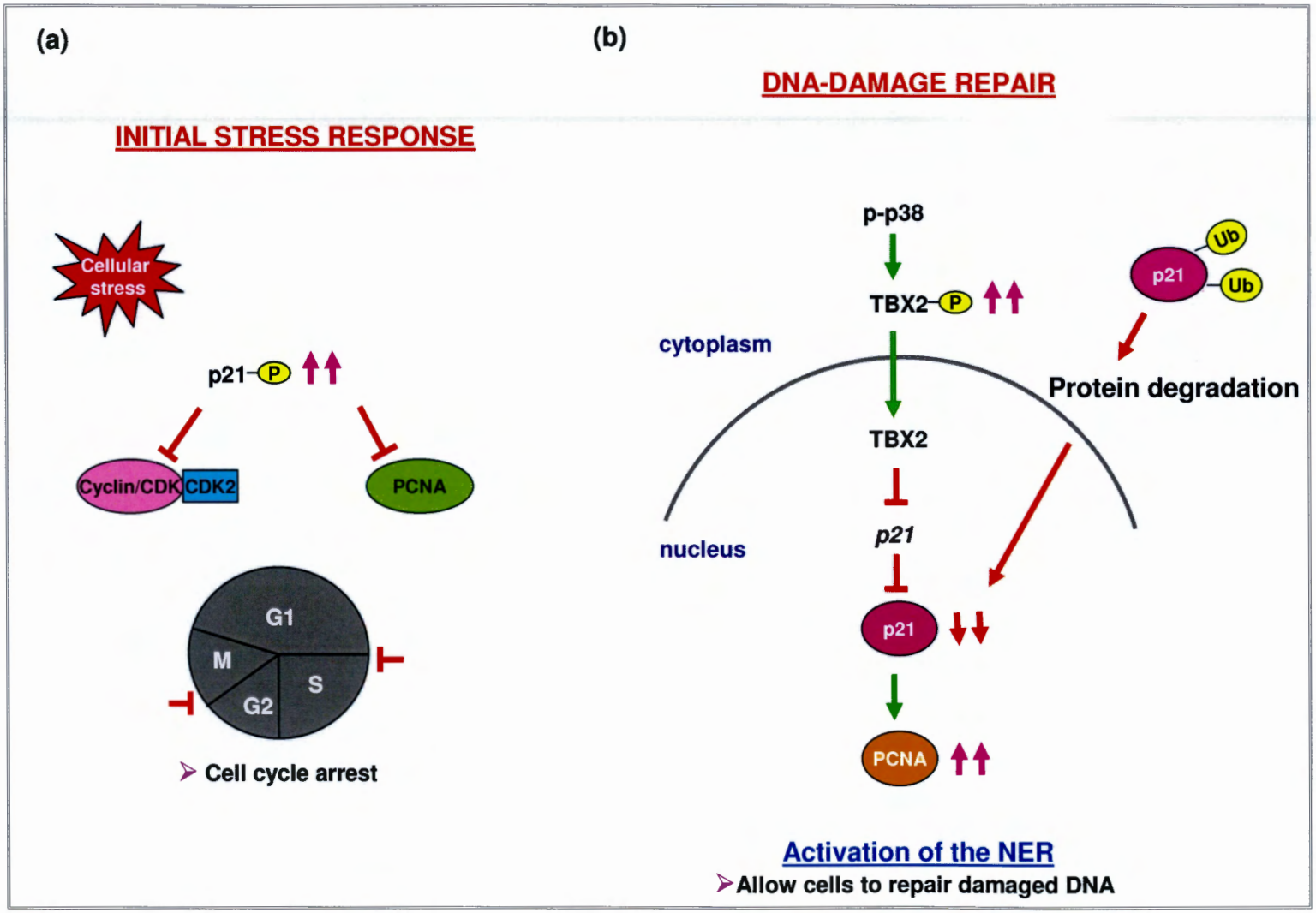


**Figure 4.1.** Possible model for the mechanism by which TBX2 represses *p21* in response to stress-induced DNA damage. (a) Established model for the role of p21 in response to cellular stress. (b) Suggested model for the role of TBX2 in the DNA damage pathway.

the effect of silencing *TBX2* by siRNA on *p21* levels in the presence of UVC irradiation. This would involve transfecting *TBX2* siRNA into MCF-7 cells, exposing the cells to UVC irradiation and then using real-time PCR to analyse the expression of *p21*. It is anticipated, that if TBX2 is responsible for keeping the *p21* gene repressed that *p21* mRNA levels should increase.

It is important to note that since these studies were performed in a breast cancer cell line that overexpresses TBX2 it is possible that the effects seen on *p21* may be due to TBX2 inappropriately inhibiting *p21* in response to DNA damage. This would result in cells cycling with damaged DNA which could lead to genomic instability, which is a hallmark of cancer. It would therefore be important to repeat the above experiments in a breast epithelial cell line expressing normal levels of endogenous TBX2.

**The cell cycle regulated TBX2 is a target for cyclin A/Cdk2 and cyclin B1/Cdk1**

Several lines of evidence have implicated a role for TBX2 in the cell cycle. For example, it was shown to act as an anti-senescence factor capable of repressing the Cdki's, $p19^{ARF}$ and *p21* (Jacobs et al., 2000; Lingbeek et al., 2002; Prince et al., 2004). Furthermore, a role for TBX2/Tbx2 at G2 and/or early M has been suggested by studies that have shown that its protein levels are tightly regulated during the cell cycle with levels peaking at G2 (Bilican and Goding, 2006) and ectopic expression of TBX2 leads to several mitotic defects and genomic instability (Davis et al., 2007; Demay et al., 2007). This study shows that the increase in TBX2 levels previously seen during G2 is due to phosphorylation. The same result was obtained in the mouse B1Tbx2 fibroblast cell line, which stably expresses exogenous Tbx2. An interesting observation was that in contrast to the mouse cell lines used in this study, phosphorylated TBX2 persists at high levels in human cells synchronised in M-phase. These results allude to the possibility that TBX2 may be regulated differently in cell lines of human and mouse origin and hence that it may have different roles in the cell cycle in these two species. While these results are intriguing, many more cell lines of human and mouse origin would have to be analysed to test this possibility. Moreover, it is important to point out that the regulation of TBX2 levels during the cell cycle must be due to post-translational modifications because exogenous TBX2 protein levels in CT-Tbx2 and B1Tbx2 are driven by constitutively active viral promoters and yet behave similar to the human and mouse endogenous TBX2 protein respectively.

It was hypothesised that the increased levels in phosphorylation of TBX2 at G2/M imply that it is a substrate for the cyclinA/Cdk2 and/or cyclin B1/Cdk1 complexes, which phosphorylate proteins required for the events of G2 and M. The present study provides evidence to suggest that this is the case. Firstly, when B16 mouse melanoma and CT-Tbx2 human fibroblast cells were treated with olomoucine, a Cdk1 and Cdk2 inhibitor, a dramatic decrease in Tbx2/TBX2 protein levels at G2 and M in mouse and human, respectively was observed. These results suggest that the increased levels of TBX2/Tbx2 at G2 and M are due to phosphorylation by Cdk1 and/or Cdk2. Secondly, TBX2 was shown to be exclusively nuclear in G2 cells and this localisation appears to be regulated by phosphorylation by Cdk1 and/or Cdk2, since in the presence of olomoucine the protein does not enter the nucleus. Other studies have also shown that phosphorylation by the cyclin/Cdk complexes regulates the subcellular localisation of its

target substrates. For example, phosphorylation of CDC6, a key regulator in the initiation of DNA replication, by cyclin A/CDK2 induces the translocation of the protein from the nucleus to the cytoplasm to prevent re-replication of the DNA (Peterson et al., 1999). The results presented in the present study show for the first time that phosphorylation of TBX2/Tbx2 by cyclin A/Cdk2 and/or cyclin B1/Cdk1 regulates the levels and subcellular localisation of the protein in a cell cycle dependent manner.

The above results raised the question as to whether TBX2/Tbx2 is phosphorylated and regulated by both cyclin A/Cdk2 and cyclin B1/Cdk1 or by only one of the two. It was postulated that since cyclin A/Cdk2 and cyclin B1/Cdk1 are both active at G2/M it was unlikely that they would target the same substrate. Interestingly, not only was Tbx2 shown to bind both cyclin A and cyclin B1 but it was also shown to be phosphorylated by both cyclin/Cdk complexes. However, while the two kinases share a common target site in Tbx2, they each also have a second distinct target site. The identified cyclin A/Cdk2 and cyclin B1/Cdk1 sites were also shown to be phosphorylated in vivo as mutating these serine residues abolished phosphorylation of Tbx2. Although limited, there is some evidence in the literature to suggest that both Cdk1 and Cdk2 in association with the appropriate cyclin can indeed bind and differentially phosphorylate a common substrate. For example, an investigation by Santaguida and Nepveu, (2005) show that the homeodomain protein, CDP/Cux, is differentially phosphorylated by cyclin A/Cdk2 and cyclin A/Cdk1. While phosphorylation by cyclin A/Cdk2 allows CDP/Cux DNA-binding during S phase, phosphorylation by cyclin A/Cdk1 was shown to inhibit the DNA-binding and transcriptional activity of CDP/Cux in G2. Although it is generally accepted that Cdk1 and Cdk2 recognise a common consensus sequence, it has been shown that any variation from this sequence can affect the specificity and kinase activity of the Cdk towards its potential substrates (Holmes and Solomon, 1996). It is therefore possible that variation in the sites adjacent to the SP motifs within TBX2 may lead to its differential phosphorylation by cyclin A/Cdk2 and cyclin B1/Cdk1, which may have different effects on the function of the protein.

Cyclin A and cyclin B1 were both shown to bind TBX2 within its DNA-binding domain, which suggests that these two cyclins may compete for binding to TBX2. Interestingly, results previously generated in our laboratory showed that unlike the S192A mutant, the Tbx2 S192E mutant which mimics phosphorylation by cyclin A/Cdk2 was unable to bind cyclin B1/Cdk1 in vivo. The present study explored the functional significance of TBX2 phosphorylation by cyclin A/Cdk2 and cyclin B1/Cdk1. While the results obtained for cyclin A/Cdk2 were inconclusive, the positive effect of cyclin B1/Cdk1 on TBX2 protein stability and transcriptional activity was reproducibly demonstrated. It is therefore possible that phosphorylation of TBX2 by cyclin A/Cdk2 serves to prevent inappropriate stabilisation and transcriptional activity of the TBX2 protein due to premature binding and phosphorylation by cyclin B1/Cdk1. This theory is supported by data generated by our UK collaborators who showed that when cyclin A was knocked down by siRNA in human cells, TBX2 levels increased. It is also worth noting that in the mouse cells tested in the present study, the downregulation of Tbx2 in M-phase corresponds with the absence of cyclin B1. This further supports the notion that phosphorylation by cyclin B1/Cdk1 is responsible for stabilising Tbx2. To resolve this issue it would be important to knock-down

cyclin B1 and determine the consequence on TBX2 levels. In the event that TBX2 levels remain high, both cyclin A and B1 may need to be knocked down together to determine whether they are both capable of regulating TBX2 levels and can substitute for one another. Furthermore, given the limitations of the use of olomoucine in distinguishing between phosphorylation by Cdk1 and Cdk2, it would be important to test the effect of an inhibitor specific for either Cdk2 or Cdk1 on TBX2 phosphorylation during the cell cycle.

The results in this study which show that pseudo-phosphorylation of Tbx2 by cyclin B1/Cdk1 enhances the ability of Tbx2 to repress *p21* raised the question of the potential significance of this effect. It was speculated that TBX2 may function to repress *p21* in order to allow cells to progress into mitosis. While p21 is generally regarded as a negative regulator of the cell cycle, it was recently shown to play a positive role in G2/M progression. At G2/M, a transiently hyperphosphorylated form of p21 binds cyclin B1 to promote the assembly and activation of the cyclin B1/Cdk1 complex (Dash and El-Deiry, 2005). However, immediately after activating cyclin B1/Cdk1, the hyperphosphorylated form of p21 was shown to be rapidly downregulated to promote progression into mitosis. The mechanism for this was, in part, recently elucidated by Amador et al. (2007) who showed it involves the ubiquitin ligase $APC/C^{Cdc20}$. Results from the present study would suggest that in addition to p21 protein degradation, phosphorylation of TBX2 by cyclin B1/Cdk1 may also downregulate *p21* mRNA levels.

One of the most unexpected results to emerge during this study was the difference in the expression of cyclin A and B1 during the various phases of the cell cycle in the mouse and human cell lines tested. While it is widely accepted that cyclin B1 in association with Cdk1 plays a critical role in the early phases of mitosis, cyclin B1 was undetectable in the mouse, but not human cells, synchronised in M-phase. In the mouse cells high levels of cyclin A were however observed in M-phase suggesting that cyclin A may substitute for cyclin B1 to complex with Cdk1. This is not unlikely since both cyclin A and cyclin B1 have been shown to be capable of associating with Cdk1 and Cdk2 (reviewed in Kaldis and Aleem, 2005; L'Italien et al., 2006). The absence of cyclin B1 in M-phase in mouse cells cannot be explained by a lack of specificity of the cyclin B1 antibody because the antibody recognises cyclin B1 in all the other phases of the cell cycle. This effect on the downregulation of cyclin B1 has previously been observed in polyploid megakaryocytes, which fail to undergo complete mitosis (Zhang et al., 1996, 1998). The ubiquitin-dependent degradation of cyclin B1 was postulated to contribute to the genesis of polyploidy by abrogating mitosis in these cells (Zhang et al., 1998). While B16 mouse melanoma cells are polyploidy tumour cells (Baroja et al., 1996), early passage B1Tbx2 cells did not display features of polyploidy (our unpublished data). However, based on previous published data from our laboratory that show that ectopic expression of TBX2 leads to polyploidy (Davis et al., 2007), it is possible that with increasing passage in culture these cells may indeed become polyploid and hence metaphase spreads would need to be performed on later passage cells.

In conclusion, the present study has identified the stress-responsive p38 MAP kinase and the cell cycle regulatory complexes, cyclin A/Cdk2 and cyclin B1/Cdk1 as novel regulators of

TBX2. The results generated for the p38 kinase demonstrate that TBX2 may be required to inhibit *p21* mRNA levels in order to enable DNA damage repair in response to UV irradiation. The results showing that phosphorylation of TBX2 by cyclin A/Cdk2 and/or cyclin B1/Cdk1 regulates the stability and subcellular localisation of the protein provides additional support for a role for TBX2 in G2 and/or early M.

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# APPENDIX

2.2 *Site-directed mutagenesis of the mouse Tbx2 cDNA*

NYZ$^+$ Broth

10 g NZ amine
5 g yeast extract
5 g NaCl

Adjust to pH 7.5 using NaOH
Autoclave
Add the following supplements prior to use:
12.5 ml 1M $MgCl_2$
12.5 ml 1M $MgSO_4$
10 ml of 2 M filter-sterilised glucose solution or
20 ml 20% (w/v) glucose
Filter sterilize

2.3.2 *Mycoplasma test*

Mounting fluid

1.1 M Citric acid
1.2 M $Na_2HPO_4.2H_20$
100% glycerol

Adjust to pH 5.5
Aliquot and store at 2-8°C

2.4.2 *Cell cycle synchronisation and Flow cytometry*

Phosphate Buffered Saline (PBS)

8 g NaCl
1.45 g $Na_2HPO_4.12H_2O$
0.2 g KCl
0.2 g $KH_2PO_4$

Adjust to pH 6.9, make up to 1 litre and autoclave

Propidium iodide stain

0.1% Triton X-100
0.002 M $MgCl_2$
0.1 M NaCl
0.01 M PIPES
0.01 mg/ml propidium iodide

Cover with foil and store at -20°C

2.4.4 *Cycloheximide treatment*

2X SDS loading buffer

125 mM Tris-HCl (pH 6.8)
4% SDS
2% glycerol
2-4 mg bromophenol blue
10% β-mercapto-ethanol

Aliquot and store at 4°C

2.4.5 *Phosphatase treatment*

5X SDS loading buffer

0.25 M Tris-HCl (pH 6.8)
1% SDS
10% glycerol
2-4 mg bromophenol blue

Aliquot and store at RT

2.9 *Western blot analyses*

RIPA buffer

150 mM NaCl
1% Triton X-100
0.1% SDS
10 mM Tris-HCl (pH 7.5)
1% deoxycholate

Store at 4°C

Supplement with 1 mg/ml aprotinin, 1 mg/ml Pepstatin A and 2 mM phenylmethanesulfonyl fluoride protease inhibitors (Sigma, USA) just before use.

Blocking solution

5% non-fat dry milk dissolved in 0.1% Tween-20 in PBS

2.9 *Bacterial expression and purification of glutathionine S-tranferase (GST) fusion proteins*

PBSTi Buffer

1% Triton X-100
1X PBS

Supplemented with 4 complete proteinase inhibitor tablets (Roche, Switzerland)
Store at -20°C

2.10 *In vitro kinase assay*

Kinase buffer

20 mM MOPS (pH 7.2)
25 mM β-glycerophosphate
5 mM EGTA
1 mM sodium orthovanadate
1 mM dithiothreitol

Store at -20°C

[γ-$^{32}$P]ATP (Amersham Biosciences, USA)
10 µM Ci diluted with 9µl of 400 µM unlabelled ATP, 75 mM $MgCl_2$

2.11 *In vitro transcribed translated binding assays*

In vitro transcribed translation reaction

1 µg Tplink DNA
40 µl TNT T7 Quick master mix
3 µl [$^{35}$S] methionine (1.175Ci/mmol at 10 µCi/µl)
2 µl 25X EDTA-free proteinase inhibitor tablets (Roche, Switzerland)

Make up to a final volume of 50 µl with nuclease-free $H_2O$

2.12 *GST-pulldown assays*

RIPA 150 mM NaCl buffer

0.05 M Tris-HCl (pH 8)
0.15 M NaCl
0.1% NP-40
0.1% sodium deoxycholate
0.1% SDS
5 mM EDTA
1 mM DTT
10 mM NaF
0.01 mM sodium orthovanadate
2 complete proteinase inhibitor tablets (Roche, Switzerland)

Store at -20°C

2.13 *Quantitative reverse transcription PCR (qRT-PCR)*

ΔΔ*Ct* method

Formula ratio = $(Etarget)^{CPtarget(control-sample)}/(Eref)^{CPref(control-sample)}$; E: real-time PCR efficiency, CP: crossing point (Pfaffl, 2001).